

# **ISOLATION AND CHARACTERIZATION OF ENTEROCOCCI FROM CLINICAL SAMPLES IN A TERTIARY CARE HOSPITAL**

Dissertation submitted to  
**The Tamil Nadu Dr. M.G.R. Medical University**  
In partial fulfillment of the regulations  
For the award of the degree of  
**M.D. MICROBIOLOGY**  
**Branch – IV**



**DEPARTMENT OF MICROBIOLOGY**  
**PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH,**  
**PEELAMEDU, COIMBATORE, TAMILNADU, INDIA**

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**CERTIFICATE**

This is to certify that the dissertation work entitled **“Isolation and characterization of Enterococci from clinical samples in a tertiary care hospital”** submitted by Dr.Mohamadiya Rizwana .M., is the work done by her during the period of study in this department from April 2014 to August 2015. This work was done under direct guidance of Dr. S.Parvathi, Professor, Department of Microbiology, PSGIMS & R.

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## **Acknowledgement**

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First, I want to thank ALLAH for giving me the strength to carry out my project work successfully. I also thank my parents for helping me throughout my journey and my father Mohamed Ali in being a moral support to me to pursue this course; my mother Pareena for her patience and prayers.

My heartfelt gratitude to the Dean, Dr.S.Ramalingam, who had permitted me to carry out the work in the department and supported at all levels.

I would like to take this opportunity to extend my deep gratitude to Dr. B. Appalaraju, Professor & Head, and Department of Microbiology, who enhanced my learning and enlightened my vision in Microbiology. He was the guiding light for me professionally.

With deep sense of gratitude, I acknowledge the kind help rendered by my guide Dr. S.Parvathi, for having guided at every level. I thank her for taking keen interest in this study and time she has spent at every stage in spite of her very busy schedule.

I thank faculty, technicians and staff of Microbiology who encouraged me to give my best. It gives me immense joy to thank my colleagues Dr.Saikeerthana , Dr.Uma Maheswari and Dr.Lavanya for their constant support and understanding. It was indeed my pleasure interacting and working with them.

I would like to place on record my gratitude to my husband Arshad and my in-laws for being a constant pillar of strength and support to me at all times.

I am obliged to thank my grandparents and aunt for their constant blessings.

Special thanks to my little daughter Faizah for her sacrifice of tolerating my absence.

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## ABSTRACT

**INTRODUCTION:** *Enterococci* mainly being commensals in human faeces are now considered as an important cause of nosocomial infections. Of the infections, the commonly observed are urinary tract infections, abdominal infections followed by bacteremia, endocarditis and meningitis rarely. The initial treatment for enterococcal infections has become challenging due to development of resistance. The resistance has been observed mainly against aminoglycosides due to the presence of bi-functional gene. VRE strains are mostly isolated from patients with recurrent bacteremia, endovascular infections leading to increase deaths in the patients. Limited treatment options are available for drug resistant strains.

**AIM:** To isolate and speciate *enterococcal* isolates from clinical samples, study the antibiotic susceptibility pattern and the genotype associated with the aminoglycoside and vancomycin resistance. To screen the high risk patients in critical care areas for VRE from stool samples.

**MATERIALS AND METHODS:** *Enterococcus* isolates obtained from various sections were characterized by conventional phenotypic methods. The antibiotic susceptibility was studied by disc diffusion method. The resistant strains were further confirmed by MIC using automated methods following which the genotypic analysis was done. Screening of VRE carriers from high risk group was done using rectal swabs and results interpreted after inoculation in chrome agar.

**RESULTS & CONCLUSION:** The incidence of *enterococci* was 2.5% in which around 86.4% were obtained as pure isolates. Among the species *E.faecalis* was the maximum. The urinary isolates exhibited sensitivity of around 92.3% to nitrofurantoin and among the non - urinary isolates the maximum sensitivity was for linezolid followed by vancomycin and other antibiotics. The molecular study showed van A to be most common gene with vancomycin resistance(71.42%), and bifunctional gene among the aminoglycoside resistance(96%).The incidence of VRE carriers was 3%.The increase in resistance patterns in hospital strains reflects the limited treatment options that might pose a risk in future in managing complicated cases.

**KEYWORDS:** Enterococci, speciation, antibiotic resistance, vancomycin, aminoglycosides, genotype, vancomycin resistant *enterococci*(VRE) carriers.



## Introduction

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*Enterococci* mainly being commensals in human faeces are now considered as an important cause of nosocomial infections. Of the infections, the commonly observed are urinary tract infections, abdominal infections followed by surgical site infections, bacteremia, endocarditis and meningitis rarely. They are referred to as the third organism in causing nosocomial infections in the west.<sup>1</sup>

*Enterococci* being the commonest micro flora of both humans and animals share their source of origin .They have been isolated from variety foods including cheese, fish, sausages, beef and pork. Studies have been reported that enterococci have been isolated from various clinical and environmental samples all over the world. Among the isolates obtained clinically it has been observed that *Enterococcus faecalis* has been the predominant species clinically.<sup>2,3</sup>

*Enterococcal* infections have been referred to as tough, tenacious and troublesome infections.<sup>4</sup> In the past years there has been an increase in the prevalence of *enterococcal* infections in hospitals and of particular concern is the emergence of antimicrobial resistant strains. Being second commonest organism behind the abdominal and pelvic infections, third in causing blood stream infections, cases have also been reported in causing CNS and neonatal infections.

Reports have been confirmed with their association with clinical conditions including respiratory tract infections, osteomyelitis and cellulitis.<sup>5</sup> *Enterococci* was initially regarded as the disease causing agent in the early nineties. But now

various reports have been established reporting them as the second commonest pathogen.<sup>6</sup>

*Enterococci* separated as gram positive cocci initially were later classified under the genus *Streptococcus*. Based on a series of biochemical tests and Lancefield serological typing enterococci were classified as group D streptococcus separating them from non-enterococcal group D streptococci.<sup>7,8</sup> In 1930s, Sherman recommended the term “*enterococcus*” to be used specifically for the streptococci species that had the following characteristics, growth at 10 and 45 degree Celsius, pH 9.6, 6.5% NaCl and survival at 60 degree for 30 minutes. They also had the ability to hydrolyze esculin with 40% bile. In 1980, they had their own genus *enterococcus*.<sup>9</sup>

Species identification becomes essential for investigating outbreaks in case of nosocomial infections and also susceptibility pattern varies accordingly which is also helpful in clinical management of infections due these agents.<sup>10</sup>

Infections caused by *enterococcal* pathogens were initially being treated with the combined treatment of cell wall active agents as penicillins and aminoglycoside as the synergistic effect was effective. But now due to the development of antibiotic resistance as high level aminoglycoside resistance, beta lactamase production and also resistance to vancomycin have led to serious concern in management of these nosocomial pathogens.<sup>11</sup>

Resistance spread among the *enterococcal* isolates in hospitals has led to treatment failure. Also the excess use of antibiotics and the pressure due to colonization play a major role in the drug resistance in these species.<sup>12</sup>

Vancomycin resistant enterococcus was initially reported in 1980s , after which there was a increase in the incidence of its spread .The main phenotypes associated with its spread are Van A, Van B and Van C. Van A and Van B are mainly associated with *E.faecalis* and *E.faecium*.Van C is noted in *E.gallinarum* and *E.casseliflavus*. Among the phenotypes Van A is predominantly associated in the resistant strains.

VRE strains are mostly isolated from patients with recurrent bacteremia, endovascular infections leading to increase deaths in the patients. Vancomycin resistance and penicillin resistance are often seen co-existing which makes the treatment harder.<sup>1</sup>

Resistance development in enterococcus against glycopeptide antibiotics gets transferred from them to other bacterial pathogens which include *Staphylococcus aureus*, *Streptococci*, *Listeria monocytogenes*.<sup>13</sup>

Thus due to the recent emergence in association with significant clinical infections and development of resistance, enterococci have gained importance. Studies are thus required especially in tertiary care hospitals for appropriate isolation, identification and speciating them. Antibigrams should also be followed up in accordance to the species. Emergence of VRE needs to be checked and limit its spread in hospital environment. CDC also stresses the

control of VRE in hospitals by educating the staff in detecting VRE early, reporting them and action plan taken promptly.

My study aims to isolate, speciate the *enterococcus* in a simplified manner and also to study the drug sensitivity and resistance patterns among the isolates obtained.

## **Review of literature**

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*Enterococcus* is also referred to as the 'streptococci of fecal origin'. The term 'Enterocoque' was used for the intestinal origin of these gram positive bacteria occurring in pairs and short chains which was published in a paper presentation in France in 1899. In the same year a case of endocarditis was reported which was believed to be caused by *Micrococcus zymogenes*. Later studies demonstrated it to be hemolytic *Enterococcus*.<sup>14</sup>

Enterococcal species have gained importance by becoming one of the most common pathogens in causing infections associated with urinary tract, blood stream, CSF and infections associated with soft tissues. With the involvement of resistance to clinically significant drugs as vancomycin and aminoglycosides has increased their importance due to the risk of spread of resistance to other sensitive strains.

In 1906 the term *Streptococcus faecalis* was used for the same. Fermentation of mannitol and lactose by these organisms was also demonstrated in them. *Streptococcus faecium* was demonstrated in 1919 which differed from other species in their biochemical reactions. *Streptococcal durans* was proposed in 1935 whose fermentation reactions showed variations.<sup>15</sup> *Enterococcus* was grouped into those gram positive organisms that grew at 10 and 45 degrees, pH 9.6 and 6.5% NaCl, also survived heating for 30 minutes at 60 degree.<sup>4</sup>

The above classification also matched Lancefield's serological scheme that was developed in 1930 such that it reacted with group D antisera.<sup>16</sup>

*Streptococcus avium* was added to the group in 1967. In the year 1970 a separate genus on the basis of cellular arrangement of *Streptococcus faecalis* and *faecium* was recommended to be named as *Enterococcus*.<sup>15</sup>

Discussions on *enterococcus* was also done by various authors.<sup>17</sup> It was in the year 1984 *Streptococcus* was divided into *Enterococcus*, *Lactococcus* and *Streptococcus*. Based on the studies done a large number of species were included in the genus accordingly. The various species included in this genus were *E.avium*, *E.durans*, *E.casseliflavus*, *E.gallinarum* *E.faecium* *E.faecalis*, *E.hirae*, *E.malodoratus*, *E.mundtii* based on the DNA-DNA hybridization. New species as *E.raffinosis* and *E. solitarius* were also discovered.<sup>18</sup> Motile enterococci discovered initially were called *Streptococcus faecium subspecies mobilis* was renamed as *Streptococcus faecium var casseliflavus*.<sup>19</sup>

Biochemical and genetic data shows that the strains *S.casseliflavus*, *S.durans*, *S.faecalis sub sp.malodoratus* and a few others have close relations to the members of *Enterococcus* as described initially. Studies revealed that the above mentioned species were renamed under genus *Enterococcus* as *E.avium*, *E.casseliflavus*, *E.durans*, *E.malodoratus* and *E.gallinarum* respectively.<sup>20</sup> In 1985 *E.hirae*, a new species was identified.<sup>21</sup> *E.solitarius* was also identified from human sample.<sup>22</sup> *E.rattus* was isolated from the intestine of neonatal diarrheal rats and thus named accordingly. *E.porcinus* was identified from pigs.<sup>23</sup> Recently new species *E.gilvus* and *E. pallens* were identified in the year 2002.<sup>24</sup> *E.phoeniculicola* was also included under the genus.<sup>25</sup>



## **TAXONOMY:<sup>26</sup>**

Kingdom : Bacteria

Phylum : Firmiculates

Class : Bacilli

Order : Lactobacillales

Family : Enterococcaceae

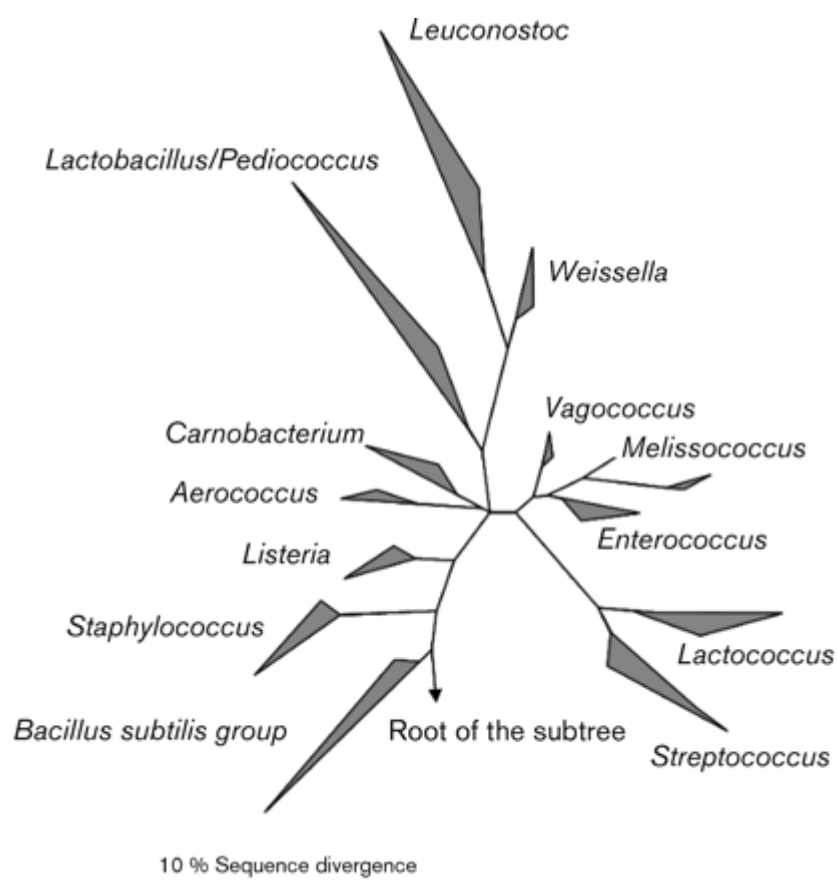
Genus : Enterococcus

## **GENUS DEFINITION AND TAXONOMY:**

Enterococci as a genus hydrolyze esculin in the presence of 40% bile. They are catalase negative and are fermentative with lactic acid as the end product of glucose fermentation without the production of any gas. Majority of the strains about 95% have the group D antigen in their cell wall. Pyrrolidonyl - 3-naphthylamide (PYR) hydrolysis is a special feature of this genus. DNA content is 38-45% of these genus. About 28 species are currently included under them. *Enterococcus faecalis* is the type species of this genus.<sup>15</sup>

Molecular information gathered with the help of 16S RNA sequencing explained the relationship between the related species. The grouping of the species was also based on this method. A dendrogram showing the phylogenetic position of Enterococcus was made.<sup>27</sup>

## 16S rRna DENDROGRAM OF PHYLOGENETIC POSITION OF ENTEROCOCCUS SPECIES:



### HABITAT:

Enterococci are found everywhere in the environment and survives harsh conditions. They are found in the plants, soil, water, animals and food. Also found in the birds, insects and reptiles. In human beings they are mainly inhabitants of intestinal tract followed by the genitourinary and mouth.<sup>15</sup> Their concentration in stool varies from  $10^5$ - $10^7$  CFU/gm of faeces. Studies have been published suggesting that *Enterococcus faecalis* is observed in high numbers than others in the faecal samples.<sup>28</sup> The other species were isolated from the intestinal tracts of various animals and unusual causes of infections in humans.<sup>26</sup>

## MORPHOLOGY AND CULTURAL CHARACTERISTICS:

Enterococcus are gram positive bacteria occurring in pairs and short chains measuring around,  $0.6 - 2 \times 0.6-2.5\mu\text{m}$ . With the exceptions of *E.casseliflavus* and *E.gallinarum* other species are non-motile. Being facultative organisms enterococci have the ability to grow at a temperature range of 10 to 45 degree and survives heating at 60 degree for 30 minutes.<sup>29</sup> Growth in blood agar shows colonies ranging from 1-2 mm in diameter or smaller. *E.faecalis* is non haemolytic in sheep blood agar but can vary to haemolytic form when rabbit, horse or human blood is used.<sup>26</sup>

The alpha hemolytic property of enterococcus species is mainly due to the peroxide production of the species. The greenish discoloration of the blood agar is due to the peroxide action and not because of the synthesis of alpha toxin. The pigment production of certain species including *E.casseliflavus*, *E.gilvus*, *E.pallens*, *E.mundtii* and *E.sulfureus* is also observed.<sup>15</sup> In Mac Conkey agar tiny (0.5-1mm) magenta pink colonies are seen due to lactose fermentation. Their growth is also well appreciated in media with increased salt content i.e. 6.5% NaCl which aids in differentiating it from others.<sup>30,31</sup>

Growth has also been noted in selective media which includes bile esculin agar in which colonies are observed greyish surrounded by black halo that occurs as a result of hydrolysis of esculin to esculitin that reacts with ferric ammonium citrate to give black colour.<sup>15</sup> Other special medias employed are Pfizer-selective enterococcus agar, oxoline-esculin agar, selective media with

vancomycin for VRE screening.<sup>27</sup> Enterococci being aerobe and facultative anaerobe need certain growth requirements which includes methionine, histidine, tryptophan and isoleucine but some require other amino acids as glutamate, glycine and leucine.<sup>15</sup>

### **SPECIES IDENTIFICATION:**

Enterococcal species causes a wide range of clinical diseases. Speciation is not mandatory for all Enterococcal infections. The majority of the isolates are associated with urinary tract infections in which case a genus level identification along with antibiotic sensitivity with antimicrobials including Penicillin, Ampicillin and Vancomycin is satisfactory. In conditions like endocarditis, speciation is necessary mainly between *E.faecalis* and *E.faecium* for the purpose of treatment. *E.faecium* is noted to be more resistant to Penicillin group and most of the clinically isolated VRE (Vancomycin resistant enterococci) are *faecium* species. The speciation depends on a wide range of biochemical reactions which includes production of acid from sorbitol, glycerol, D-galactose, melibiose, L-arabinose. Certain special characteristics are exhibited by few species characteristically which includes motility by *E. casseliflavus* and *E. gallinarum*. Pigment production by *E.casseliflavus* and *E. mundtii*.<sup>18</sup>

It is necessary to identify the motile species because they have the feature of being intrinsically resistant to antimicrobials as Vancomycin at low levels and if improperly treated can lead to complications and unresponsiveness in patients. The tests apart from motility to identify *E.gallinarum* and *E.casseliflavus* are

pigment production and failure to ferment inulin. These are mainly associated with infections in immunocompromised and organ transplant patients.<sup>32</sup>

Enterococcus are classified as gram positive, catalase negative organisms with positive L-pyrrolidonyl beta-naphthylamide (PYR), LAP (Leucine aminopeptidase), and presence of growth at 45 degree and 6.5% NaCl.

The species are classified into 5 groups which are as follows:<sup>26</sup>

Group 1	<i>E. avium, E. gilvus, E. malodoratus, E. raffinosus E. pallens, E. saccharolyticus E. pseudoavium</i>
Group 2	<i>E. casseliflavus, E. gallinarum E. faecalis, E. faecium, E. mundtii, E. haemoperoxidus</i>
Group 3	<i>E. dispar, E. durans, E. hirae, E. ratti, E. villorum</i>
Group 4	<i>E. asini, E. cecorum, E. sulfureus, E. phoeniculicola</i>
Group 5	<i>E. canis, E. columbae, E. moraviensis.</i>

**Identification of enterococcal species presumptively is based on:**<sup>30</sup>

- 1) Catalase test
- 2) Salt tolerance test
- 3) Heat tolerance test
- 4) Bile esculin test
- 5) PYR test
- 6) Arginine test
- 7) Carbohydrate /Sugar fermentation test
- 8) Potassium tellurite test (0.04%)

## 9) Vogues – Prosker (VP) test

**1) Catalase test:** Enterococci do not possess the enzyme cytochrome oxidase thus are catalase test negative on testing with hydrogen peroxide. Few of the strains do produce delayed reaction categorizing them as pseudo catalase producers.<sup>15</sup>

**2) Salt tolerance test:** When colonies are inoculated in 6.5% NaCl broth, increase in turbidity after overnight incubation indicates positive test.<sup>30</sup> Modifications in the form of rapid test has been proved to be effective in species isolation.<sup>31</sup>

**3) Heat tolerance test:** In 1914, studies have demonstrated heat tolerance to be one of the characteristic feature exhibited by enterococcus that helps it in being differentiated from other species.<sup>16,26</sup> It survives heating for 30 min at 60 degree distinguishing it from other Streptococcal species.<sup>30</sup>

**4) Bile esculin hydrolysis:** This test proved to have a sensitivity of 100% and specificity of 97% .<sup>15,33</sup>

**5) PYR test:** (Hydrolysis of L- pyrrolidonyl beta – naphthlamide test): This test was used as a presumptive identification for Enterococci and group A beta haemolytic streptococci. Initially the test was 20 hour test but now 4 hours incubation in PYR broth at the end of which PYR reagent added gives the result.<sup>34</sup>

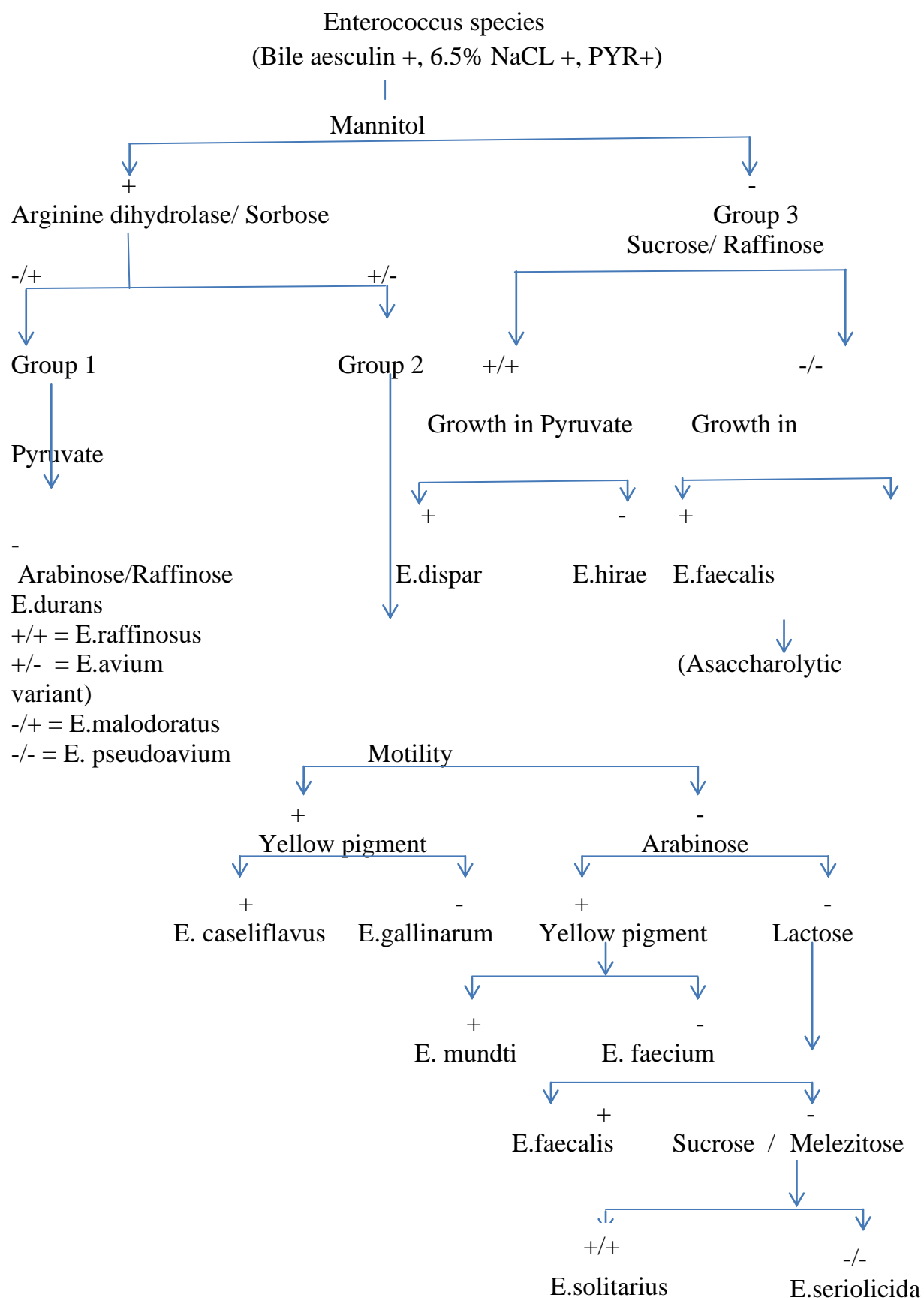
**6) Arginine test:** Studies have proved that hydrolysis of arginine was used as a dependable test in species identification.<sup>19</sup>

**7) Carbohydrate fermentation test:** A wide range of carbohydrates as lactose, sucrose, raffinose, sorbitol, maltose, trehalose, arabinose, mannitol, inulin, salicin were used for speciation.<sup>18,19</sup> Initially 26 tests were done for speciation by which *E.faecium*, *E.avium*, *E.casseliflavus*, *E.equinus* were isolated.<sup>17</sup> Later other tests as sorbose fermentation, pyruvate utilization and arginine deamination were added.<sup>17,18</sup> In 1989, Facklam and Collin presented a simplified scheme for the identification of species through conventional tests.<sup>18</sup>

**8) Potassium tellurite test:** Studies showed that when 0.04% of potassium tellurite was added to the agar, a few of the enterococcal species had the property to reduce tellurite forming black colonies.<sup>17,18</sup>

**9) Voges- Proske (VP) test:** Tests were done on enterococcus for testing the production of acetoin and comparing with rapid Strep system results.<sup>35</sup> Various methods were compared and Coblatz method proved to be the best in differentiation of the species.<sup>30</sup>

## ALGORITHM FOR IDENTIFICATION OF ENTEROCOCCI





Commercial systems are now available for the identification of Enterococcal species which includes API 20S (Analytab Products, Plainview, N.Y), GPI (Gram positive identification) , Vitek Systems Inc., Hazelwood, Mo.), the Rapid Strep System (API). The advantages of these systems is that they provide the result faster than the biochemicals that consume more time provided enough inoculum is present. Studies have reported that species as *E.faecalis*, *E. faecium*, *E.avium* and *E. durans* are identified properly.<sup>36,37,38</sup>

Molecular studies as in DNA hybridization also confirmed the genetic variations between the *E.faecalis* and *E.faecium*. Estimated that approximately 6.4% of this genome is in conjunction with cell – surface proteins and about 22.6% of the differences between the species are observed in these genes.<sup>27</sup>

## **PHYSIOLOGY:**

*Enterococcus* is a highly resistant bacteria and can even grow in conditions when the temperature, pH and salt concentration is increased. The effect of temperature is mostly observed in the lag phase of the growth cycle while the stationary phase being more resistant to it.<sup>39</sup> The resistance of *Enterococcus* to pH , is believed to be due to the durability and impermeability of the cell membrane to acid and alkali.<sup>40</sup> The resistance nature depends on both the phase of growth and temperature of the species.<sup>41</sup> The amine production is also been associated with temperature, growth and pH .Studies suggest that decarboxylase activity is observed at acidic pH but this is not favourable for the

production of biogenic amine. The amine production is not affected by temperature directly but its effect on the cell affects the production of amine.<sup>39</sup>

## **ECOLOGY AND EPIDEMIOLOGY:**

The occurrence of *Enterococcus* species varies widely from animal, human and environmental sources. They form a part of microflora in human intestinal tract (*E.faecalis* and *E.faecium*), where as *E.faecium* is predominant in animals whereas *E.mundtii* and *E.casseliflavus* is observed in plants.<sup>42</sup>

The distribution of *Enterococcus* varies widely throughout Europe. In the UK, they have been observed in both the environmental and also the clinical samples from the patients. Sweden shows an increased rate of *E.hirae* similar to Denmark.<sup>43</sup> Studies from India suggest that *E. faecium* and *E.faecalis* are the predominant species in India.<sup>44</sup>

The *Enterococcus* species have become the second causative organisms of hospital infections<sup>20</sup> and the third causative organism in United States.<sup>45</sup> *Enterococci* show a low level resistance to antimicrobials as penicillin, cephalosporins, aminoglycosides and lincosamides.<sup>46</sup> Attention to these species recently is mainly due to the resistance pattern demonstrated by them to a wide range of antibiotics mainly aminoglycosides, ampicillin and vancomycin. Beta lactamase production is also been reported among these species.<sup>47</sup>

## **VIRULENCE FACTORS AND PATHOGENESIS:**

The virulence factors seen in *Enterococcus* based on the various studies are:<sup>27,48-51</sup>

- 1) Cytolysin/ Haemolysin
- 2) Aggregation substance
- 3) Extracellular surface protein
- 4) Lipoteichoic acid
- 5) Cocco lysin
- 6) Gelatinase
- 7) Extra cellular superoxide
- 8) Biofilm
- 9) Pili
- 10) Microbial surface components recognizing adhesive matrix molecules (MSCRAMM)
- 11) Cell wall and Capsular polysaccharide
- 12) Glycolipids

**Cytolysin:** *Enterococci* possessing this factor has the ability to cause lysis of horse, human and rabbit erythrocytes. This is believed to be more virulent in animals than human beings.<sup>52</sup> The cytolytic action is due to the post translationally modified protein toxin seen in almost 60% of *faecalis* isolates.<sup>53</sup> Experiments in rabbits have demonstrated that endocarditis due to *Enterococcal*

species with cytolytic action can result in increased mortality in comparison with those strains without it.<sup>54</sup>

**Aggregation substance:** It causes the efficient donor – recipient contact resulting in effective plasmid transfer through conjugation. It is actually a pheromone inducible protein located on the surface of *E.faecalis* that results in mating. The various functions associated with this substance are adhesion to extracellular matrix (ECM) protein, attachment to host cells and increasing the hydrophobicity of the cell surface. Studies have demonstrated that in vitro that prior immunization of rabbits with these substances does not provide any protection against endocarditis caused due to them. Studies have confirmed that its role in pathogenesis is due to evasion of immune system.<sup>52</sup>

**EXTRA CELLULAR SURFACE PROTEIN:** This is a cell-wall associated protein seen in the virulent strains believed to originate from *E.faecalis* with Van B genotype. It is encoded by the esp gene present on the surface of the bacteria. In conditions associated with immunodeficiency it retracts the surface protein that results in further immune evasion.<sup>55</sup>

**LIPOTEICHOIC ACID:** It forms the group D antigen of the Enterococcal species. It causes the production of tumour necrosis factor and also interferon resulting in modulation of immune response.

**COCCOLYSIN:** It constitutes the extracellular metalloendopeptidase that inactivates the vasoactive peptide endothelin.<sup>26</sup>

**GELATINASE:** Enterococci producing gelatinase are found to be associated with peritonitis, endocarditis and endophthalmitis in rabbits. It is an extracellular zinc metalloprotease that hydrolyses gelatin, casein and collagen.<sup>56</sup> This property is encoded in an operon which is up regulated by quorum sensing by the *fsr* which is a regulator locus.<sup>48</sup> This property can be demonstrated in vitro by inoculating Enterococci in freshly made yeast extract agar plates with gelatin and incubating at 37 degree overnight followed by cooling for two hours. Positive activity is denoted by a halo around the colonies.<sup>52</sup>

**EXTRA CELLULAR SUPEROXIDE:** It is an important factor associated with *enterococcal* infections causing bacteremia.<sup>49</sup> This feature provided the survival advantage of *E.faecalis* along with *Bacteroides fragilis* in mixed infections.<sup>57</sup>

**BIOFILM:** The production and adherence of *Enterococcal* species due to biofilm formation on various materials have been demonstrated. There appears to be a direct relation between the production of biofilm and its ability to bind with stents, catheters, silicon gastroenterology devices and other medical devices. Various studies have been put forth regarding the prevalence of biofilm production among *Enterococcal* species. Studies report that about 80% *E.faecalis* and 48% *E. faecium* are associated with biofilm production isolated from infected patients.<sup>58</sup> A wide range of factors including the presence of glucose, serum, availability of CO<sub>2</sub> and iron, osmolarity, pH have an effect over the production of biofilm in these virulent strains. The carbohydrate metabolism

influences the development of biofilm among various gram positive cocci especially *E.faecalis*. Studies have shown that 1% glucose supplemented with trypticase soy broth(TSB) helps in the demonstration of biofilm better than media without it. It has been proved that glucose dependant regulator (fsr) is responsible for biofilm production. Apart from fsr other factors such as Enterococcal surface protein (esp), secretory metalloprotease gelatinase (gel E), biofilm associated pili and biofilm enhancer are proved to produce biofilm.<sup>59</sup>

**PILI:** The presence of pili in Enterococcus is responsible for the adhesion of the bacteria to human cells and result in disease. There are genes that encode these pili as PGC (pilin gene clusters) that are necessary for its assembly. These along with the presence of biofilm formation results in the pathogenesis of experimental endocarditis and also urinary tract infections. Studies have been stated that expression of these pili gene is more in *E. faecium* than *E.faecalis* species.

**MSCRAMM:** Microbial surface components recognizing adhesive matrix molecules play an important role in the colonization of Enterococcus. Among the species *E.faecalis* claimed to have 17 and *E.faecium* had 15. But only 7 among them are studied in detail. These include:

- a) Ace(adhesion of collagen in *E.faecalis*)
- b) Fss 1, Fss 2, Fss 3(*E.faecalis* surface protein)
- c) Acn(adhesin of collagen of *E.faecium*)
- d) Scm(second collagen adhesion of *E.faecium*)

e) EcbA(*E.faecium* collagen binding protein A)

The presence of these virulence factors have increased the severity of infections caused by them. Of these Ace was seen in endocarditis patients experimentally. EcbA was observed in nosocomial infections due to *E.faecium*.<sup>51</sup>

**CELLWALL AND CAPSULAR POLYSACCHARIDE:** The recognition of polysaccharide antigen on the bacterial surface (Epa) by patients with systemic enterococcal infections has been demonstrated in various studies. Much information is not available for its structure and just that is has been localized inside the cell wall.<sup>60</sup> This is directly related to the pathogenicity as any alteration results in reduction in biofilm formation.<sup>61</sup>

**GLYCOLIPIDS:** The presence of glycolipids plays an important role in the pathogenesis of clinical infections. They form a permeable layer between the environment and cytoplasm. One glycolipid in particular DGlc-DAG is present mainly in *E.faecalis* constituting about 37% of the polar lipids present in the cell membrane. The mutant incapable of producing this glycolipid could not produce the disease.<sup>51</sup>

## **INFECTIONS CAUSED BY ENTEROCOCCI:**

*E.faecalis* and *E.faecium* are the two species predominantly associated with clinical infections. Studies have been reported suggesting that *E.faecalis* accounts for around 80 to 90% followed by *E. faecium* that comprises of about 15% of the infections identified.<sup>15</sup> Other species that can cause infections include

*E.casseliflavus*, *E. avium*, *E.durans*, *E.gallinarum*, *E.casseiflavus*, *E.hirae*, *E.pseudoavium*, *E.raffinosis*, *E.solitarius*, *E.dispar*, *E.malodoratus*, *E.saccharolyticus*, *E.serioicida*, *E.cecorum*, *E.flavescens*. The infections caused by these species include urinary tract infections, endocarditis, intraabdominal and intra pelvic abscesses, blood stream infections and wound infections following surgery. Occasionally they are also isolated in central nervous system and neonatal infections. Cases have been reported them to be causative organisms in osteomyelitis and respiratory infections.

### **URINARY TRACT INFECTIONS:**

Enterococci are found to be the most common cause of infections involving the urinary tract. Infections range from uncomplicated to complicated UTI s involving cystitis, prostatitis, pyelonephritis and perinephric abscess. Most of these infections have been reported in hospitalized individuals.<sup>62</sup> Studies show that the percentage of significant bacteriurea of urine sample cultures which is  $> 10^5$  has increased from 11% to 20% in catheterized individuals than in midstream urine cultures.<sup>63</sup> The Center for Disease Control's National Nosocomial Surveillance Study rates enterococci as being the third common cause of nosocomial UTI.<sup>1</sup>

### **INTRA- ABDOMINAL AND PELVIC INFECTIONS:**

Enterococci form part of the intestinal flora normally and account for around 17% of vaginal cultures in the female. Enterococci are important pathogens associated with intra-abdominal infections and sepsis. Cases have also



been reported indicating them to be the causative organisms in spontaneous peritonitis in patients with cirrhosis and nephrotic syndrome. It also results in peritonitis in patients on long term dialysis.<sup>64</sup> They have also been associated with salphingitis, maternal infections as endometritis and post Cesarean abscess formation.<sup>65</sup>

### **ENTEROCOCCAL BACTEREMIA:**

Bacteremia due to enterococcus accounts to be the third most common cause of blood stream infections.<sup>8</sup> The most common source of infection is endocarditis and if not its urinary tract infections. Studies suggest that patients had underlying source of infection as urinary or intravascular catheter, abdominal or pelvic surgery, burns, and peripartum maternal infections when presenting with bacteremia.<sup>66-69</sup> Mortality rate is high which may be related to the underlying risk factors.<sup>1</sup>

### **ENTEROCOCCAL ENDOCARDITIS:**

Enterococci accounts for nearly 15% of endocarditis due to bacterial causes. The main pathogen is *E.faecalis* but other species also play a role in this manifestation. Individuals above 65 years are more prone to enterococcal endocarditis.<sup>1</sup> The other risk factors involved are proceeding UTI's or catheterization, biliary infections. Studies have been reported suggesting that 38% of women had prior genital infection or instrumentation. It can present as acute or chronic condition. It's mostly associated with an underlying heart

disease but this does not form a prerequisite.<sup>70</sup> Enterococcal endocarditis has also been reported in drug addicts. The valves mainly affected are mitral and aortic.<sup>71</sup>

### **NEONATAL INFECTIONS:**

Group B streptococci and *Escherichia coli* are the main pathogens causing infections in neonates, but it has been recorded that Enterococcal species can also cause infections in this age group. Nosocomial spread is strongly associated with these infections.<sup>1</sup> Infants with these infections had a remarkable feature of previous premature delivery, low birth weight and some cases long standing central line was recorded.<sup>72</sup>

### **CENTRAL NERVOUS SYSTEM INFECTION:**

Apart from neonates enterococcal species can cause meningitis in older children and adults rarely. Most of them have a prior risk factor as an invasive procedure or long term infection and on prolonged antibiotic.<sup>1</sup> Cases have been reported them to be associated with brain abscess. The effect is mainly due to the haematogenous spread from a localized infective site as sinusitis or otogenic origin.<sup>73</sup>

### **NOSOCOMIAL INFECTIONS AND SUPERINFECTIONS:**

Studies have proved that enterococcal species to be the major cause of infections acquired from the hospitals.<sup>74</sup> The recent studies proved that person – to – person transfer being the important source. It's been noted that treatment with antimicrobials without enterococcal activity has led to superinfections.

The use of polymyxin aerosol in the prevention of pneumonia associated with *Pseudomonas* has led to the spread of Enterococcal induced respiratory infections. Studies from the west have reported the increase in the infection rate with liver transplant patients.<sup>1</sup>

### **SHIFTING SPECTRUM OF ENTEROCOCCAL INFECTIONS:<sup>8</sup>**

The ratio of *E.faecalis* to *E.faecium* was nearly 10:1. But this ratio has been declining. The change has been remarkably noted in bacteremia infections in which there is a decrease in the ratio from 3.7:1 to 1.9:1. The *E.faecium* is found to be increasing in these isolates. Now there has been a rise in the VRE in which *E.faecium* species dominates. Studies have reported a 47% increase in the VRE isolates. The cause of concern is the emergence of pan-resistant species that show resistant to ampicillin, vancomycin, gentamycin and streptomycin.

### **PHENOTYPIC RESISTANCE:**

This type of resistance includes intrinsic resistance of Enterococci to penicillins, cephalosporins, lincosamides, nalidixic acid, low level aminoglycosides, low level clindamycin and cotrimoxazole.<sup>4</sup>

The acquired resistance includes the resistance acquired genetically.

### **GENETIC MECHANISM:**

The resistance among the enterococcal species varies. It has been observed that *E.faecalis* has low level resistance to ampicillin and vancomycin compared to *E.faecium*. Gene responsible for resistance can be carried on the

pheromone and be transferred between the two species. The motile species account for less than 2% but have the property of intrinsic resistance. These species have found to be associated with infections in immunocompromised individuals.<sup>8</sup> Conjugative gene transfer occurs by spread of transposons between gram positive and gram negative bacteria. Low frequency transfer has been noted from *E.faecalis* and *Listeria* species in gnotobiotic mice.<sup>75</sup> The other mechanism of transfer of genetic matter is through phages but spread of virulence strains is not recorded.<sup>76</sup>

### **SENSITIVITY TESTING:**

The antimicrobials used in testing against enterococcus include ampicillin, penicillin, vancomycin, amoxycillin clavulanic acid, erythromycin, high level gentamycin and linezolid. In case of urinary isolates norfloxacin, nitrofurantoin and tetracycline is used along with the above and penicillin is not used.<sup>77</sup> Initially MIC s for ampicillin and penicillin as 8(moderately sensitive) and 16(resistant)µg/ml was reported but recent CLSI (2014) suggests ≤8 as sensitive and not moderately sensitive.<sup>1,77</sup> But for vancomycin MIC ≤4 ,8-16, ≥32 µg/mg is considered sensitive, moderately sensitive and resistant respectively. If disc is used (30µg disk) a zone of ≥17, 15-16, ≤14 is interpreted as sensitive, moderately sensitive and resistant respectively.<sup>77</sup>

Isolates from patients with endocarditis and life threatening infections as meningitis, aminoglycoside activity is to be determined. MIC of greater than 2000µg/ml proves HLR.<sup>1</sup>

### **1) High- content disc:**

Studies were reported stating that the use of high content aminoglycoside disc were effective for screening HLR enterococcal species.<sup>78</sup> Comparative studies for MIC determinants on Muller- Hinton plates using high content gentamycin disc(120µg) and tobramycin along with tobramycin(300µg) was done.<sup>1</sup> Further studies used gentamycin and kanamycin with 120µg along with 300µg of streptomycin. Strains without HLR activity had zone size  $\geq 12$ . The disc diffusion method was able to detect all the gentamicin resistant strains but only 94% of streptomycin resistant strains.<sup>79</sup>

### **2) Agar screening:**

Most of the laboratory usually does not perform MIC or synergy testing as a routine, other screening test for HLR is sought. Inoculation of enterococcal colonies in dextrose phosphate agar with 2000µg of aminoglycoside per ml was effective in detecting the presence or absence of synergy.<sup>80</sup> Studies further proved that irrespective of the media used with both gentamycin and streptomycin; good results were obtained when spot inoculated with  $10^4$ - $10^6$  CFU per spot.<sup>81</sup>

### **3) Broth dilution:**

In this method only one concentration of aminoglycoside in micro dilution well was used. Studies were done using 500µg of gentamycin in Muller- Hinton broth along with Ca and Mg with an inoculum of  $5 \times 10^5$  CFU/ml.<sup>82</sup>

### **Recommendations for HLGR to aminoglycosides:<sup>1</sup>**

- 1) Single concentration agar plate and the disk diffusion are highly for detecting HLR to gentamycin and streptomycin.
- 2) Broth dilutions are acceptably good except that there is a possibility to miss a few strains with streptomycin 2000µg/ml.
- 3) Caution must be employed on using automated systems as comparison with standard methods is not evaluated.
- 4) To test for aminoglycosides apart from gentamicin and streptomycin is of little significance as most of the data is based on these. Strains with HLR(high level resistance) to gentamicin alone without HLR to streptomycin due to enzymatic action of 2''-APH-6'-AAC is observed.

### **TYPING METHODS:<sup>15</sup>**

Typing for Enterococci has become essential as there has been a rise in the number of clinical infections caused by them. Adding on to this is the resistance pattern exhibited by these species which justifies the epidemiological purpose in studying these isolates by typing them.

#### **Phenotypic methods of typing:<sup>83</sup>**

- 1) Bio typing
- 2) Antibiotyping
- 3) Serotyping and
- 4) Bacteriophage typing.

The above methods proved to be of limited value in typing the species in comparative studies.<sup>84</sup>

### **Molecular methods of typing:<sup>85-87</sup>**

- 1) Ribotyping
- 2) Pulse field gel electrophoresis.(PFGE)
- 3) Restriction enzyme analysis. (REA)
- 4) Polymerized chain reaction (PCR)

Of the above methods PFGE is considered as the gold standard in epidemiological study of enterococcus.<sup>15</sup>

### **ANTIMICROBIAL RESISTANCE:**

Resistance in enterococci is of two types' intrinsic/inherent resistance and acquired resistance. The intrinsic resistance of these species is chromosomal in nature. Resistance to penicillin (low level), cephalosporins, nalidixic acid, lincosamides, low level clindamycin and low level aminoglycoside has been noted.<sup>4</sup>

#### **1) Resistance to beta lactams:**

Resistance is exhibited by one of the following mechanisms:

- a) Intrinsic resistance to beta lactam antibiotic
- b) Tolerance to beta lactams
- c) Beta lactamase production

**a) Intrinsic resistance:** This property is mainly exhibited due to the low affinity of penicillin binding protein PBP-5. In comparison of *E.faecalis* which has an MIC of 1 to 8 µgm/ml to penicillin; *E.faecium* has higher MIC of about 16-64 µgm/ml.<sup>4,12</sup>

**b) Tolerance to beta lactams:** This is exhibited by the species in being inhibited but not killed by the presence of these antimicrobials. Tolerance is shown by most of the enterococcal species which justifies the usage of combination drugs as cell wall active agents along with aminoglycosides in treatment of serious infections rather than using monotherapy.<sup>8</sup>

**c) Beta lactamase production:** Enterococci producing beta lactamase with higher MIC to ampicillin and penicillin have been isolated. The amount of beta lactamase produced is little and may go undetected by routine antibiotic screening in laboratories. Enterococcal infections from serious infections have to be tested for beta lactamase activity. The method recommended for its detection is cephalosporin (nitrocefin) impregnated disc. Isolates with this property is susceptible only in combination with clavulanic acid, tazobactam and sulbactam.<sup>88</sup>

## **2) Resistance to clindamycin:**

Studies have been reported in enterococcal resistance against Clindamycin and Lincomycin. The MIC of these strain vary from 12.5 to 100µgm/ml.<sup>89</sup>



### 3) Resistance to aminoglycosides:

Resistance is of two types<sup>4</sup>

- a) Intrinsic resistance.
- b) Acquired resistance.\

**a) Intrinsic resistance:** Enterococcus species have a low level of resistance to aminoglycosides intrinsically. The MIC's range from 8-256µgm/ml. This is overcome by the synergy effect by combination of beta lactam antibiotic with an aminoglycoside. This effect is mainly useful to treat long standing serious infections.

**b) Acquired resistance:** Synergistic effect of enterococci was affected when the organism developed high level resistance to streptomycin initially.<sup>90</sup> This HLR was subsequently observed to gentamicin and penicillin combination.<sup>91</sup>

The high level resistance is defined when the isolates have an MIC >2000µgm/ml for streptomycin and MIC >500µgm/ml for gentamicin. The resistance is mainly brought about due to the presence of plasmid mediated aminoglycoside modifying enzyme. The most commonly observed enzymes are:

- a) 2'phosphotransferase and 6'acetyl transferase observed in all aminoglycoside usage except streptomycin.
- b) 3' phosphotransferase observed in HLR to penicillin-amikacin synergy and kanamycin without gentamycin.

c) 6' adenylyl transferase having HLR to streptomycin only. The methods involved in studying the high level resistance include agar screening, broth dilution, high content disc and broth dilution method.<sup>4</sup>

#### 4) Resistance to glycopeptides:

Resistance to glycopeptides mainly vancomycin was reported in 1988 in the UK first.<sup>92</sup> The peptidoglycan layer of the enterococcus is cross linked with the help of a pentapeptide chain at the D-ala ends. Vancomycin sensitive strains are attacked at the cross linking stage and its disrupted thus affecting its stability. The resistant strains have D-lac at the ends instead of D-ala thus overcoming the action of vancomycin and preserving the cross linkage.<sup>93</sup> Phenotypically the vancomycin resistant strains are classified into 6 types: Van A, Van B, Van C, Van D, Van E and Van G. Of these Van A and Van B are more commonly associated with *E.faecalis* and *E.faecium*.<sup>15</sup>

**TABLE.2:**<sup>3</sup>

Characteristic	Phenotype				
	VanA	VanB	VanC	VanD	VanE
Vancomycin MIC (µg/ml)	64–>1,000	4–1,024	2–32	128	16
Teicoplanin MIC (µg/ml)	16–512	≤0.5	≤0.5	4	0.5
Most frequent enterococcal species	<i>E. faecium</i> , <i>E. faecalis</i>	<i>E. faecium</i> , <i>E. faecalis</i>	<i>E. gallinarum</i> , <i>E. casseliflavus</i> , <i>E. flavescens</i>	<i>E. faecium</i>	<i>E. faecalis</i>
Genetic determinant	Acquired	Acquired	Intrinsic	Acquired	Acquired
Transferable	Yes	Yes	No	No	No

<sup>a</sup>Adapted in 2000 from reference 109 with permission; original version Copyright @ 1996, Massachusetts Medical Society (all rights reserved).

**1) Van A:** This shows high level resistance to both Vancomycin with a MIC of  $\geq 64 \mu\text{g}/\text{ml}$  and to Teicoplanin with MIC of  $\geq 16 \mu\text{g}/\text{ml}$ . Genotypically, van A gene is located on the transposon (Tn 1546), which is a part of the plasmid.

**2) Van B:** This shows resistance to Vancomycin with MIC of 32 to  $64 \mu\text{g}/\text{ml}$  but is sensitive to Teicoplanin. The van B gene is situated on the mobile segment of the plasmid and is interchangeable between the species.

**3) Van C:** This is associated with a low level of resistance to Vancomycin but susceptible to Teicoplanin. They are commonly seen in the motile species of Enterococcus which includes *E.gallinarum* and *E.casseliflavus*. The MIC range between 2 to  $32 \mu\text{g}/\text{ml}$ . This is intrinsic in nature unlike others which are acquired.

**4) Van D:** First isolated in a hospital in New York. This strain showed resistance to Vancomycin at a MIC of  $64 \mu\text{g}/\text{ml}$  and to Teicoplanin at  $4 \mu\text{g}/\text{ml}$ . This was found in the chromosome and is not associated with the transfer of resistance.<sup>94</sup>

**5) Van E:** Enterococcus with this gene showed low level of resistance to Vancomycin with MIC of  $16 \mu\text{g}/\text{ml}$  but sensitive to Teicoplanin with MIC of  $0.5 \mu\text{g}/\text{ml}$ . This phenotype had similarity with Van C.<sup>95</sup>

**6) Van G:** This shows low level resistance to Vancomycin with a MIC of  $16 \mu\text{g}/\text{ml}$  but sensitive to Teichoplanin.<sup>96</sup>

Studies demonstrate that mutants have emerged that have similarities between two phenotypes.<sup>3</sup>

### **VANCOMYCIN DEPENDENT ENTEROCOCCI:**

This includes those species that are not only resistant to Vancomycin but require it for its growth. This group of enterococci alters their normal production of D-Ala-D-Ala and is able to grow only in the presence of Vancomycin. This is due to the production of Van H(dehydrogenase) and ligase(Van A or Van B) that forms the D-Ala-D-Lac only if Vancomycin is present. If the Vancomycin is not present then there is no D-Ala-D-Lac and without either of these enterococci cannot survive. Reversion to Vancomycin independent form is observed which is due to mutation.<sup>3</sup>

### **VANCOMYCIN RESISTANT ENTEROCOCCI (VRE):**

Vancomycin resistant enterococci (VRE), reported initially in Europe in 1988, raised a threat to public health. Following this there was an increase in the number VRE isolates. They were associated with the raised mortality rates with no antimicrobials acting against them.<sup>47</sup> The VRE rates were found to be more in the US than other places. The geographical variation was initially thought due to the use of glycopeptide (avoparcin) as a growth promoter in animal feeds. This resulted in the cross resistance among the bacteria against vancomycin and teicoplanin. The spread of resistance was considered to be originated from animal source. But this did not prove to be an explanation for its prevalence in the US where the drug avoparcin was not licensed for animal feed.<sup>8</sup>

*Enterococci* with acquired vancomycin resistance are a major concern in the hospitals. Their importance has also increased due to the spread of its

resistance to *Staphylococcus*. This resistant species has the ability to survive for long periods and can spread to patients from health care workers. The infections caused by them are related to the immune status of the patient. Immunocompetent are not affected whereas the immunocompromised are susceptible to life-threatening infections.<sup>97</sup> The rate of VRE in India is nearly 8.7% in which the Van A phenotype is the most common.<sup>98</sup>

### **COLONISATION AND PATHOGENECITY OF VRE:**

Studies suggested that there was an increase in the number of patients colonized with VRE on screening the rectal swabs from high risk groups.<sup>99</sup> The main sites affected by VRE include urinary sites, intra-abdominal sites, surgical, blood stream and vascular sites. Infections caused by them led to serious complications and death due to bacteremia was estimated to be 60- 70%.<sup>100</sup> Studies have reported that patients with serious conditions and transplant recipients are mainly affected by VRE strains.<sup>101</sup>

Blood stream infections observed due to enterococcus are mainly catheter associated but other sites have also been reported including cerebral spinal fluid following any procedure.<sup>102</sup>

### **The risk factors with VRE colonization are:<sup>3</sup>**

- 1) Serious underlying diseases
- 2) Gastrointestinal colonization
- 3) Long duration of hospital stay

- 4) Immunosuppression as neutropenia
- 5) Patients undergoing intraabdominal or thoracic surgery with indwelling catheters.
- 6) Renal sufficiency
- 7) Usage of broad spectrum antibiotics including aminoglycosides, cephalosporins, and imipenem.

VRE carriers involve patients in the hospital, nursing faculties and other hospital staff. Spread is also observed from inanimate objects including thermometers, stethoscope, bed rails, sinks, commodes, ECG wires, intravenous fluid pumps and doorknobs.

#### **SCREENING OF VRE carriers:<sup>103</sup>**

The media used for screening of colonizers of VRE bile –esculin azide agar with vancomycin at a concentration of 6µg. This was found to be a good sensitive media for VRE isolation. Recently there has been an increase in the studies related to screening that led to the development of chromogenic media for the rapid diagnosis of the isolates in high risk groups. Studies done on the comparison of the screening media proved that the specificity was high for chromogenic media. This further had an advantage of differentiating the main species of *enterococcus*.

## TREATMENT OF VRE INFECTIONS:

Treatment of VRE infections is becoming problematic. Vancomycin resistant *E.faecalis* strains are found to have moderate susceptibility to ampicillin justifying its usage in the treatment. But if the isolates are *E.faecium* then they exhibit moderate resistance to ampicillin. The isolate is labeled ampicillin resistant if its able to grow with a drug concentration of 16µg of ampicillin per ml. If the strain isolated is resistant to vancomycin and ampicillin then additional testing of MIC should be done. Better results are obtained on combination of sulbactam with ampicillin rather than treating with ampicillin alone.

Chloramphenicol has been found to be effective against many drug resistant enterococci in vitro.<sup>3</sup> Another glycopeptide Teicoplanin has proved its efficacy against the strains that possess the Van B phenotype. Studies done on animal models with enterococcal endocarditis have proved to be effective when combined with an aminoglycoside.<sup>104</sup>

*E.faecium* strains with resistance to both vancomycin and ampicillin were found to be susceptible to the combination of beta lactam antibiotic and glycopeptide. In these strains the MIC of ampicillin is found to be decreased when present in conjunction with vancomycin. This occurs as a result of the change in the enzyme used by the species for cell wall synthesis. The MIC of ampicillin will be reduced if this new enzyme is susceptible to ampicillin.

The identification of drug effective against multiple drug resistant enterococci is under trial. Identification of Dalfopristin- quinpristin which is a streptogramin antibiotic was successful against vancomycin resistant *E.faecium* in proving its bacteriostatic effect but not active against *faecalis*.<sup>105</sup>

The introduction of oxazolidones, newer antibiotic had a good bacteriostatic effect against enterococcal species. It acts by inhibiting the translocation at the initiation of protein synthesis. Studies have proved them to be effective against multidrug resistant strains.<sup>106</sup>

### **CONTROL OF VRE:**

Studies have stressed that control of spread of vancomycin resistant enterococci plays a crucial role in preventing the spread of infections. These include justifiable use of vancomycin in treatment of infections, monitoring high risk patients on third generation cephalosporins, educating the hospital staff on the importance of infections caused and spread of VRE, effectiveness of the laboratory in early identification of carriers by appropriate screening methods and implementation of proper infection control programme in all the hospitals.<sup>107</sup>



## **Aims and Objectives**

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**AIM:**

1. To isolate and speciate *Enterococcus* from clinical samples sent to the microbiological laboratory.

**OBJECTIVES:**

1. To isolate and speciate *Enterococcus* from clinical samples sent to the microbiological laboratory
2. To study the antibiotic susceptibility pattern of the isolates.
3. To detect beta lactamase production among *enterococcal* isolates.
4. To detect the aminoglycoside and Vancomycin resistance among the isolates by genotypic methods.
5. To screen the high risk patients in critical care areas for VRE colonization from stool samples.

## **Materials and methods**

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**STUDY POPULATION:** Inpatients and outpatients of PSG Hospitals.

**STUDY LOCALE:** Diagnostic Microbiology Laboratory, PSG Hospitals.

**STUDY PERIOD:** APRIL 2014 – AUGUST 2015

**SAMPLE SIZE ESTIMATION:** 250 isolates

**SAMPLING METHOD:** Non- randomized sampling

**INCLUSION CRITERIA:** Samples from miscellaneous section, urine section and blood section

**EXCLUSION CRITERIA:** Samples from respiratory section.

**CONFIDENTIALITY:** Confidentiality of the reports were maintained

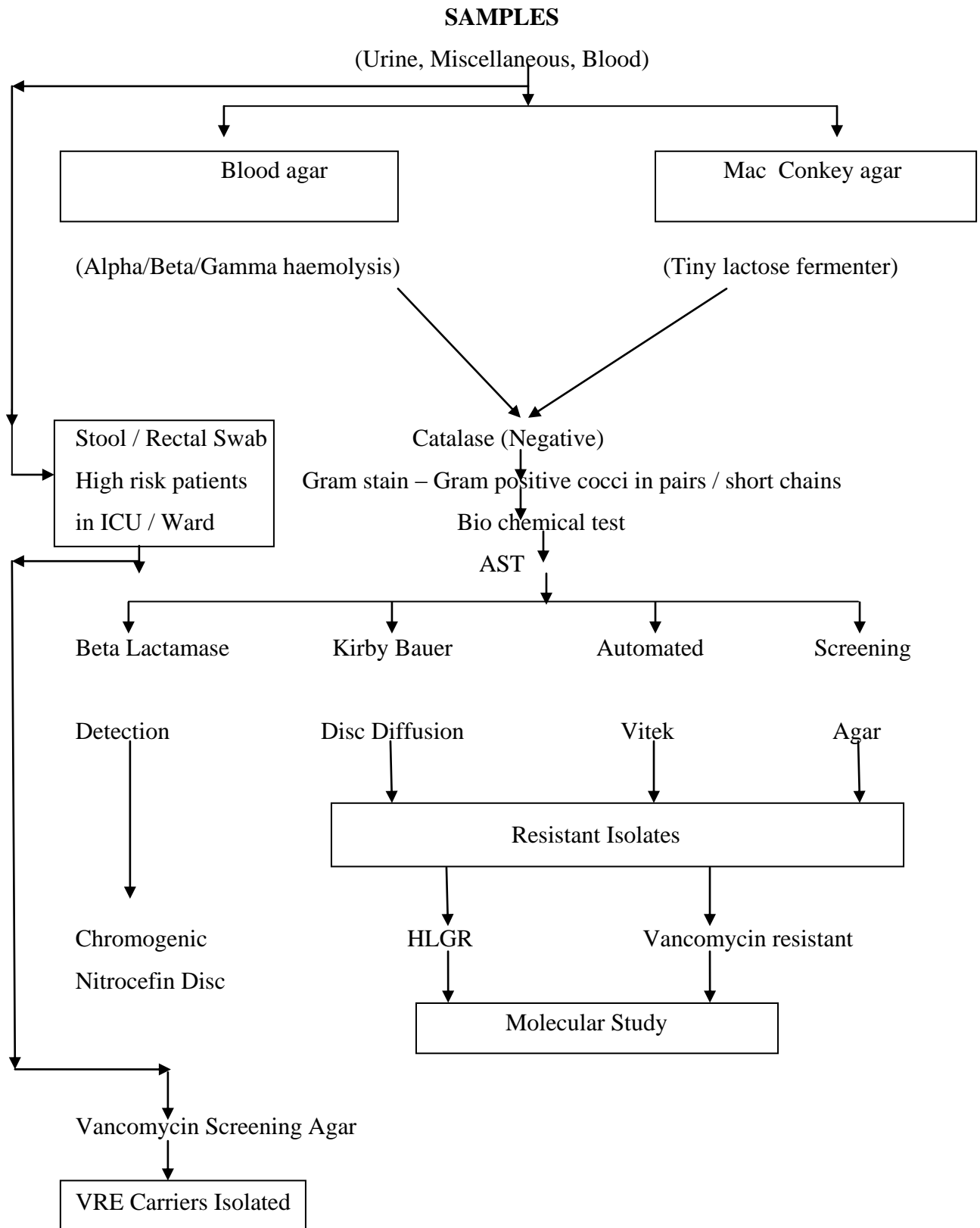
**TYPE OF STUDY:** Observational study

**ETHICAL CLEARANCE:** The Institutional Human Ethical clearance was obtained before the commencement of the study. Informed consent was obtained before collecting the faecal samples for VRE screening.

A total of 250 isolates of *Enterococci* obtained from outpatient and inpatient samples from blood section, miscellaneous and urine section were processed for the species identification, antimicrobial sensitivity followed by molecular study.

Specimen processing: Specimens from various sections including urine, pus and blood for cultures were collected and processed in the laboratory.

## FLOW CHART FOR IDENTIFICATION OF ENTEROCOCCUS



**PUS/WOUND SWABS:** The swabs were streaked in blood agar and MacConkey agar and were incubated at 37<sup>0</sup>C for 24 hours.

**URINE:** A loopful of the uncentrifuged specimen was examined as a wet mount for the presence of pus cells and bacteria. One loopful of specimen was inoculated onto blood agar and Mac Conkey agar. Plates were incubated at 37<sup>0</sup> C for 24 hours.<sup>108</sup>

**1) Growth in Sheep Blood agar:** Following overnight incubation of the samples, colonies of *Enterococcus* were seen as grey colonies of about 0.5-1mm in diameter with alpha, beta or gamma haemolysis.

**2) Growth in Mac Conkey agar:** Colonies were about 0.5- 1mm in size with a smooth surface and convex margins, tiny magenta colored following 18-24 hour growth.

**3) Catalase test:** A drop of the catalase reagent (30% hydrogen peroxide) was added to the slide. The growth was further applied to it with an applicator stick. Appearance of bubbles was taken as positive test. *Enterococcal* colonies showed a negative test due to the absence of bubbles.

**4) Gram stain:** Smears made from the colony showed Gram positive cocci arranged singly, in pairs or in short chains.

**STORAGE:** If the isolate was catalase negative, gram staining showing the presence of gram positive cocci in pairs or short chains and growth in

MacConkey agar as tiny lactose fermenting colonies, they were sub cultured and pure growth was stocked in Robertson's cooked meat medium in tubes. The tubes were sealed and stored at 4-8<sup>0</sup>C for 3-6 months. When the test was to be done they were further sub cultured.

#### **IDENTIFICATION TESTS:**

**1) Growth in media with pH – 9.6:** Isolated colonies of *Enterococcus* was streaked in a media with a pH 9.6 and incubated at 37<sup>0</sup>C overnight. The presence of growth was taken as positive.

**2) Tolerance with 6.5%NaCl:** The colonies were inoculated in a broth with 6.5% NaCl, and incubated overnight. The next day the tubes were checked for any increase in turbidity. This was further confirmed by sub culturing onto the agar plates. Growth confirmed the presence of organism. Whereas absence of turbidity indicated organism was unable to grow in this concentration.

**3) Heat tolerance test:** *Enterococcus* was inoculated in Todd-Hewitt broth. It was later incubated in water bath set at 45<sup>0</sup>C overnight and 60<sup>0</sup> C for half an hour, and then subcultured. Enterococci survived both the temperature showing positive reaction.

**4) Bile aesculin test:** Colonies from 24 hour culture were inoculated in media containing 40% bile. Blackening of the medium confirmed positive reaction for enterococci and non-enterococci.

5) **PYR test:** The colonies were inoculated in PYR broth and incubated for a period of 4 hours. Later 3 drops of PYR reagent was added to it and change of colour to red was confirmed positive indicating *enterococci* whereas absence of colour change indicates negative test.

6) **Motility test:** Stab culture of the colony was done in a semi-solid media and motility was indicated by the spread of the organism into the media. The strains which did not diffuse into the media were considered non motile. Tubes were incubated for up to 7 days for confirming motility.

7) **Arginine test:** A well isolated colony of *Enterococcus* from the culture was inoculated in Moeller's decarboxylase media with arginine as the amino acid and overlaid with mineral oil. After 24 hours of incubation the colour of the tube was changed to purple indicating a positive reaction. Yellow colour indicated only acid production only and no deamination.

8) **Potassium tellurite reduction test:** Colonies were streaked in media containing 0.004% potassium tellurite. *E.faecalis* formed black colonies with the reduction of tellurite to tellurium. This test was used to differentiate *E. faecalis* from *E.faecium*.

9) **Pyruvate test:** When inoculated in a media containing pyruvate with bromothymol blue as indicator *E.faecalis* showed a colour change from green to yellow color after overnight incubation. Absence of colour change ruled out *E.faecalis*.



10) **Pigment production:** Ability to produce pigment was observed by a few species of enterococci. This was tested by sub culturing colonies in nutrient agar. Any color produced was visible and also further confirmed by touching the colony with a sterile swab.

11) **Sugar fermentation tests:** Species identification was further done by sugar utilization by inoculating the organism in peptone with 1% sugars with Andrade indicator. The sugars used were mannitol, arabinose, raffinose, sorbose, sorbitol, lactose and sucrose. Any change in colour from colourless to pink indicated the positive sugar fermentation test. Absence of colour change was taken as negative.

#### **ANTIMICROBIAL SUSCEPTIBILITY TEST:<sup>77</sup>**

The 250 test isolates of *Enterococci* were checked for the antimicrobial susceptibility pattern using Kirby-Bauer disc diffusion method. Three or four pure colonies were inoculated in peptone water and incubated for about 3 hours. The turbidity of the growth was matched to 1 of Mc Farlands. A lawn culture of the broth with a sterile swab was done in Muller Hinton agar and the discs were placed at a distance of 24 mm. About 6 discs were kept on each plate. The discs used were Penicillin (10U), Ampicillin (10µgm), Amoxicillin Clavulanic acid (20/10), Erythromycin (15µgm), Vancomycin(30µgm), High level gentamicin(120µgm), Linezolid(30µgm), Tetracycline(30µgm). For isolates from urine section Norfloxacin (10µgm) and Nitrofurantoin (300µgm) was kept. All discs were bought from HIMEDIA.

Zones of inhibition were measured and recorded and the organism was interpreted as sensitive or resistant as per the recommendations from CLSI guidelines.

ANTIBIOTIC	DISC CONTENT (µgm)	RESISTANT (mm)	INTERMEDIATE (mm)	SUSCEPTIBLE (mm)
Penicillin	10 U	14	-	15
Ampicillin	10	16	-	17
Amoxycillin Clavulanic acid	20/10	16	-	17
Erythromycin	15	13	-	23
Vancomycin	30	14	15-16	17
High level gentamicin	120	6	7-9	10
Linezolid	30	20	21-22	23
Norfloxacin	10	12	13-16	17
Nitrofurantoin	300	14	15-16	17
Tetracycline	30	14	15-18	19

**Quality Control:** *Enterococcus faecalis* ATCC 29212 was used.

#### **SCREENING FOR HIGH LEVEL GENTAMICIN RESISTANCE.<sup>77</sup>**

Screening media for high level gentamicin resistance was prepared. Screening for high level gentamicin resistance was done by agar dilution method. BHI agar with gentamicin at a concentration of 500µgm/ml was added and the plates were prepared. Inoculum of 10µL of 0.5 Mc Farland was spot

inoculated. Presence of even 1 colony was interpreted as a resistant strain. These strains were collected for molecular studies.

#### **AUTOMATED INSTRUMENT SYSTEM FOR IDENTIFICATION & ANTIBIOTIC SENSITIVITY (VITEK 2 COMPACT SYSTEM):**

Samples were prepared and loaded into the system as per the manufacturer's instructions. About 3-4 fresh and pure isolated colonies from the blood agar plate was taken and mixed with 280µl of saline and the turbidity was matched to 0.5-0.63 of Mc Farland . The samples were placed in the plastic reagent cassette provided and loaded into the Vitek machine. After a period of 8-10 hours, identification and sensitivity report of the isolate was collected.

Fifty isolates of *Enterococci* obtained by conventional biochemical speciation were compared with the automated method (Vitek 2 system).

The Minimum inhibitory concentration of high level gentamicin resistant strains by the automated system for 50 isolates for which genetic study was to be carried out was noted. Similarly the Minimum inhibitory concentration of six isolates with vancomycin resistance by automated system was noted to detect the presence of vanA gene.

#### **DETECTION OF BETA LACTAMASE: <sup>4</sup>**

The detection of beta lactamase test was done based on the chromogenic cephalosporin method. Nitrocefin disc was obtained commercially from BD. The disc was moistened with sterile saline and pure isolated colony was applied on it.

The reaction was read within 5 min. Isolates producing beta lactamase produced a red colour.

### **SCREENING FOR VRE FROM STOOL SAMPLES:**

Screening of 100 high risk patients in the hospital Intensive Care Unit was done to identify the VRE carriers. Informed consent from the patients relatives was obtained. Rectal swabs were collected and streaked in the chromogenic VRE Chrom ID media obtained from BIOUMERIUX. The colour of the colonies was observed after a period of 48 hours of incubation. The presence of violet colour colonies indicated *E.faecium* and blue colour colonies indicated *E.faecalis*. Isolates with intrinsic resistance and most of the other gram positive organisms were selectively inhibited by the media.

### **MOLECULAR STUDY OF ISOLATES:**

Fifty isolates of *enterococcus* with resistance to high level gentamicin both by disc diffusion and by automated Vitek 2 system were studied genetically.

Similarly six vancomycin resistant isolates obtained were genetically analysed.

The colonies grown in blood agar plate were inoculated in 5ml of BHI broth and incubated for 24 hours at 37<sup>0</sup>C.

- They were centrifuged at 2000rpm for 10 minutes.

- Supernatant was discarded by pipetting and the pellet was used for DNA extraction.

### **DNA EXTRACTION:**

The DNA was extracted using the QIAGEN kit. The procedure was done based as per the manufacturer's instruction.

#### **Pre-extraction steps:**

- Absolute ethanol of about 25 ml was added to wash buffer 1(AW 1)
- Next, absolute ethanol measuring 25 ml was added to buffer 2(AW 2)
- The dry bath was set at 56<sup>0</sup> C.

#### **Extraction step:**

- Proteinase K measuring 20µl was added to a clean tube of about 1.5 ml capacity.
- About 200µl sample was added to the previous tube.
- The contents were vortexed after adding 200µl of lysis buffer.
- The contents were incubated at 56<sup>0</sup>C for 10 minutes.
- The lysate obtained was then transferred to upper reservoir of the spin column tube.
- It was centrifuged at 8000rpm for 1 minute again.
- The column was once again transferred to a new 2ml collection tube and filtered.

- Nearly 500µl of AW1 (wash buffer 1) was added and centrifuged at 800rpm for 1 min.
- The spin column tube was then transferred to a new 2ml collection tube and filtered.
- Nearly 500µl of AW2(wash buffer 2) was added and centrifuged at 14000rpm for 3 min and at 14800rpm for 1minute.
- After filtration, the spin column was shifted to 1.5ml tube.
- About 210µl of elution buffer was added and kept at room temperature for 5 minutes.
- It was then centrifuged at 8000rpm for 1 minute.
- The final eluted DNA was collected in tube of 1.5 ml capacity.

Storage of the DNA: The eluted DNA was stored at -20<sup>0</sup>C.

**PCR:** The presence of bifunctional gene for HLGR and van A gene for vancomycin resistance was detected by doing PCR and bands observed under UV illumination.

#### **DETECTION OF aac(6') le aph(2'') la gene :**

The primers for HLGR with a base pair of 348 bp were amplified. The sequence was obtained from the studies done previously<sup>109</sup>. PCR Master mix: The master mix was prepared as follows: 2.5µl of PCR buffer, 2.5µl of MgCl<sub>2</sub>, 2.5µl of DNTPs, 10.2µl of Mili Q H<sub>2</sub>O, 1µl of forward and reverse primers and

0.3µl of Taq Polymerase was added to eppendoff. From the above mixture 18µl was taken and 5µl of the extracted DNA was added.

Primers for aac(6')Ie- aph(2'')Ia with base pair of 348 bp<sup>109</sup>:

F (5'CAGAGCCTTGGGAAGATGAAG3')

R (5'CCTCGTGTAATTCATGTTCTGGC3')

**PCR Program:** The program consisted of initial denaturation step at 95<sup>0</sup>C for 10 minutes followed by 30 cycles of denaturation at 94<sup>0</sup>C for 300 seconds, annealing at 56<sup>0</sup>C for 1 minute and extension at 72<sup>0</sup>C for a period of 1 minute. The program had a holding temperature of 72<sup>0</sup>C for 10 minutes.

**ANALYSIS:** PCR products were analyzed by running gel electrophoresis with 1.5% agarose gel in Tris Boric acid buffer. The samples along with the controls obtained by running ladder were analysed. The gel was stained with ethidium bromide and the bands were obtained were visualized under UV light and also in automated GEL DOC viewer.

#### **DETECTION OF vanA gene:**

The primers with a base pair of 732bp for detection of vanA were obtained from the studies done previously.

Primers for van A with base pair of 732bp<sup>110</sup>:

F (5' GGGAAAACGACAATTGC-3')

R (5'GTACAATGCGGCCGTCGTTA-3')

**PCR Program:** This program had a denaturation step at 95°C for 5 minutes followed by 30 cycles of DNA denaturation at 95°C for 1 minute, annealing at 54°C for 45 seconds and primer extension at 72°C for 45 seconds. This was followed by a holding temperature of 72°C for 10 minutes.

**ANALYSIS:** PCR products were analyzed by running gel electrophoresis with 1.5% agarose gel in Tris Boric acid buffer. The samples along with the controls obtained by running ladder were analysed. The gel was stained with ethidium bromide and the bands were obtained were visualized under UV light and in automated GEL DOC viewer.



## Results

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All the 250 isolates of *enterococcus* from various clinical samples including urine, pus, wound swab, blood and CSF were processed.

- Gram stain showed 100% for the presence of gram positive cocci in pairs and short chains. (**Illustration 1**) and produced tiny lactose fermenting colonies on MacConkey media. (**Illustration 2**).
- **Illustration 3** shows the storage media used to preserve *enterococcal* isolates.
- **Figure 1** depicts the distribution of *enterococcal* species among the various samples received. Out of 250 samples, 184(73.6% ) were from urine section, 14(5.6% ) from blood section, 4(1.6% ) from peritoneal samples and bile, 1 (0.4% ) from CSF and 24(9.6% ) from wound samples.
- **Table 1** shows the percentage of *enterococcus* obtained as pure and mixed cultures. Majority of the isolates were obtained as pure growth which accounted for about 216(86.4%) and 34(13.6%) was obtained as mixed. Among the mixed, incidence of other organisms were in the order *Escherichia coli* was 20(58.8%), *Klebsiella pneumonia* 7(20.5% ), *Pseudomonas aeruginosa* 2(5.88% ), *Enterobacter species* 3(8.82% ) and *Candida species* 2(5.88% ).
- **Figure 2** depicts the speciation of *enterococcal* isolates. Out of the 250 isolates, 157(62.8%) were *E.faecalis*, 79(31.6% ) were *E.faecium*, 5(2%) were *E.durans*, 7( 2.8%) were *E.raffinosis* and 2(0.8%) were *E.caseliflavus*.

- **Illustration 4** shows the genus specific biochemical tests used in identification.
- **Table 2** depicts the various biochemical tests used in speciation of *enterococcus*.
- **Figure 3** shows the comparison of the manual biochemical tests with the automated Vitek system in the identification of *enterococcus*.
- **Illustration 5** shows the series of biochemical tests used in the speciation.
- **Table 3** depicts the antibiotic susceptibility pattern of *enterococcal* isolates by disc diffusion method. Out of 250 isolates ampicillin sensitive strains were 173(69.2% ), HLG sensitive strains were 123 (49.2% ), erythromycin sensitivity was observed in 59 (89.3% ), vancomycin sensitive were 243 (97.2% ), teicoplanin sensitive were 188 (75.2%).For urinary isolates, norfloxacin sensitivity was 83.15% and 170(92.39% ) strains were sensitive to nitrofurantoin.
- The maximum sensitivity was observed for linezolid followed by vancomycin, teicoplanin, ampicillin and high level gentamicin. Among the urinary isolates nitrofurantoin was observed to be sensitive in majority of the strains followed by norfloxacin and then tetracycline. Non urinary isolates had maximum sensitivity to erythromycin.
- **Illustration 6** shows the Kirby Bauer's disc diffusion test done for antibiotic susceptibility.

- **Figure 4** shows the screening of VRE carriers among the high risk patients. Out of 100 patients screened, 3% were identified as carriers.
- **Figure 5** depicts the comparison of resistance pattern obtained by disc diffusion, HLGR (high level gentamicin resistance) screening agar and Vitek in identifying resistant strains.
- **Illustration 7** shows the high level gentamicin screening agar used to identify the resistant strains.
- **Table 4** depicts the number of isolates exhibiting beta lactamase property. None of the isolates were positive for beta lactamase by chromogenic method.
- **Illustration 8** shows the chromogenic nitrocefin disc used to screen the presence of beta lactamase
- **Illustration 9** shows the chromogenic VRE screening agar used for the identification of carriers.
- **Figure 6** depicts the presence of bifunctional gene *aac (6')Ie aph(2'') Ia* in high level gentamicin resistant strains. Out of the 50 randomly selected resistant strains for genotype study 48(96%) had the gene.
- **Illustration 10** shows the presence of bands of size 348 bp in the isolates confirming the gene.

- **Figure 7** shows the presence of vancomycin resistant isolates. Out of 250 isolates 7 (2.8%) were resistant to vancomycin with 5 strains positive for van A gene.
- **Illustration 11** shows the presence of van A gene of base pair 732 confirming the presence of gene.

**TABLE 1: COMBINATIONS OF ENTEROCOCCI OBTAINED AS PURE  
AND MIXED CULTURES**

**N=250**

<b>No.</b>	<b>Bacteria</b>	<b>Percentage of isolates</b>
1	Pure <i>Enterococcal</i> isolates	86.4
2	Enterococci with <i>Escherichiae coli</i>	58.8
3	<i>Enterococci</i> with <i>Klebsiella pneumoniae</i>	20.5
4	<i>Enterococci</i> with <i>Pseudomonas aeruginosa</i>	5.88
5	<i>Enterococci</i> with <i>Enterobacter species</i>	8.82
6	<i>Enterococci</i> with <i>Candida albicans</i>	5.88

**TABLE 2: BIOCHEMICAL TESTS FOR ENTEROCOCCUS SPECIES**  
**IDENTIFICATION**

S. No	No of isolates N = 250	1 N = 157			2 N = 79			3 N = 5			4 N = 7			5 N = 2		
		P	N	%	P	N	%	P	N	%	P	N	%	P	N	%
1	Mannitol	157	0	100	79	0	100	0	5	0	7	0	100	2	0	100
2	Motility	0	157	0	0	79	0	0	5	0	0	7	0	2	0	100
3	Sucrose	0	157	0	0	79	0	2	3	40	1	6	14.2	0	2	0
4	Arabinose	0	157	0	79	0	79	1	4	20	5	1	71.4	2	0	100
5	Raffinose	0	157	0	50	29	63.2	5	0	100	7	0	100	2	0	100
6	Sorbitol	150	7	95.5	11	68	13.9	0	5	0	7	0	100	2	0	100
7	Sorbose	0	157	0	0	79	0	0	5	0	7	0	100	1	1	50
8	Arginine	157	0	100	79	0	100	5	0	100	0	7	0	2	0	100
9	Potassium tellurite (0.4 grams)	157	0	100	0	79	0	0	5	0	0	7	0	0	2	0
10	Pyruvate	140	17	89.1	0	79	0	3	2	60	3	4	42.8	0	2	0
11	Pigment	0	157	0	0	79	0	0	5	0	0	7	0	2	0	100

**1-*E.faecalis*, 2-*E.faecium*, 3-*E.durans*, 4-*E.raffinosis*, 5-*E.casseliflavus***

**TABLE 3: ANTIBIOTIC SUSCEPTIBILITY PATTERN OF  
ENTEROCOCCAL ISOLATES**

**N = 250**

<b>ANTIBIOTICS</b>	<b>SENSITIVE</b>	<b>RESISTANCE</b>
AMPICILLIN	173	77
%	69.2	30.8
HIGH LEVEL GENTAMICIN	123	127
%	49.2	50.8
VANCOMYCIN	243	7
%	97.2	2.8
LINEZOLID	247	3
%	98.8	1.2
TEICOPLANIN	188	62
%	75.2	24.8

**FOR URINARY ISOLATES    N = 184**

<b>ANTIBIOTICS</b>	<b>SENSITIVE</b>	<b>RESISTANCE</b>
NORFLOX	153	31
%	83.15	16.85
NITROFURANTOIN	170	14
%	92.39	7.61
TETRACYCLINE	147	37
%	79.89	20.11



**FOR NON - URINARY ISOLATES    N = 66**

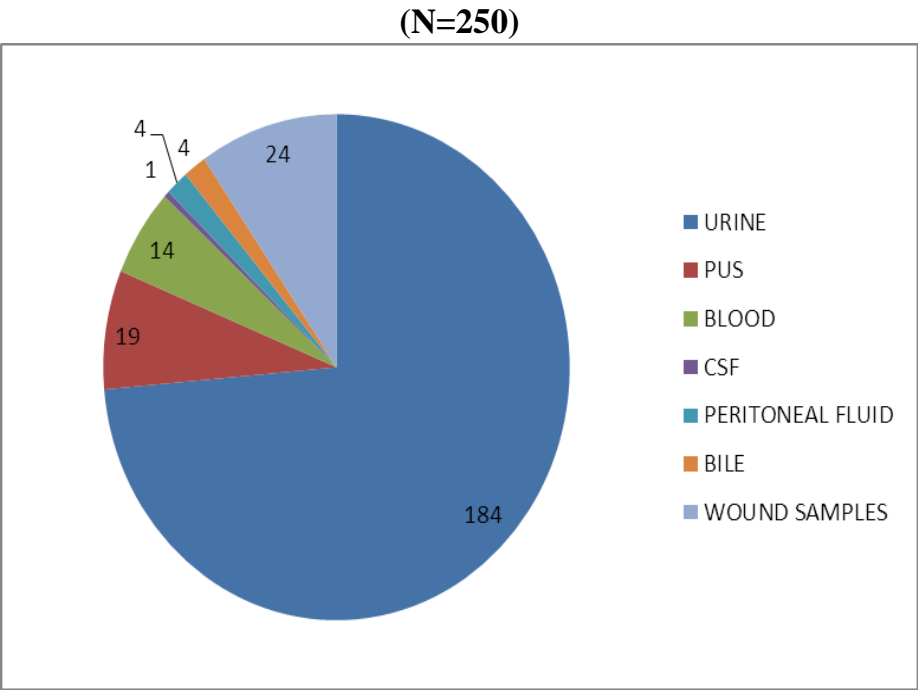
<b>ANTIBIOTICS</b>	<b>SENSITIVE</b>	<b>RESISTANCE</b>
PENICILLIN	17	49
%	25.75	74.25
ERYTHROMYCIN	59	7
%	89.3	10.7

**TABLE 4: SCREENING FOR BETA LACTAMASE ACTIVITY BY  
NITROCEFIDIN DISC**

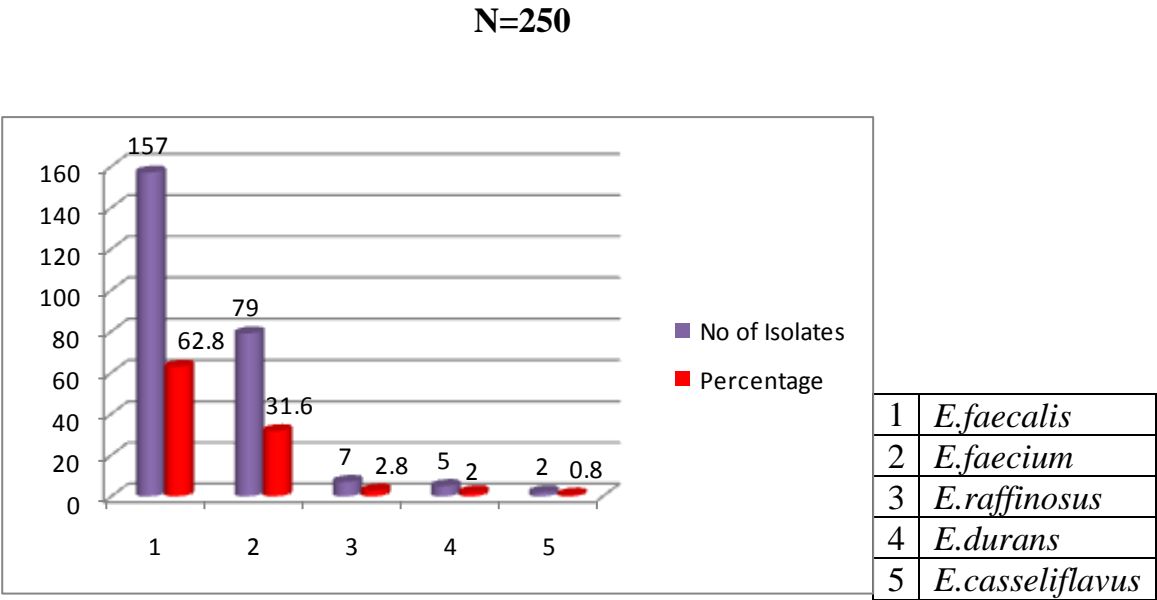
**N = 100**

	<b>POSITIVE</b>	<b>NEGATIVE</b>
N = 100	0	100

**Figure 1 – Distribution of *Enterococcus* isolates among various samples**

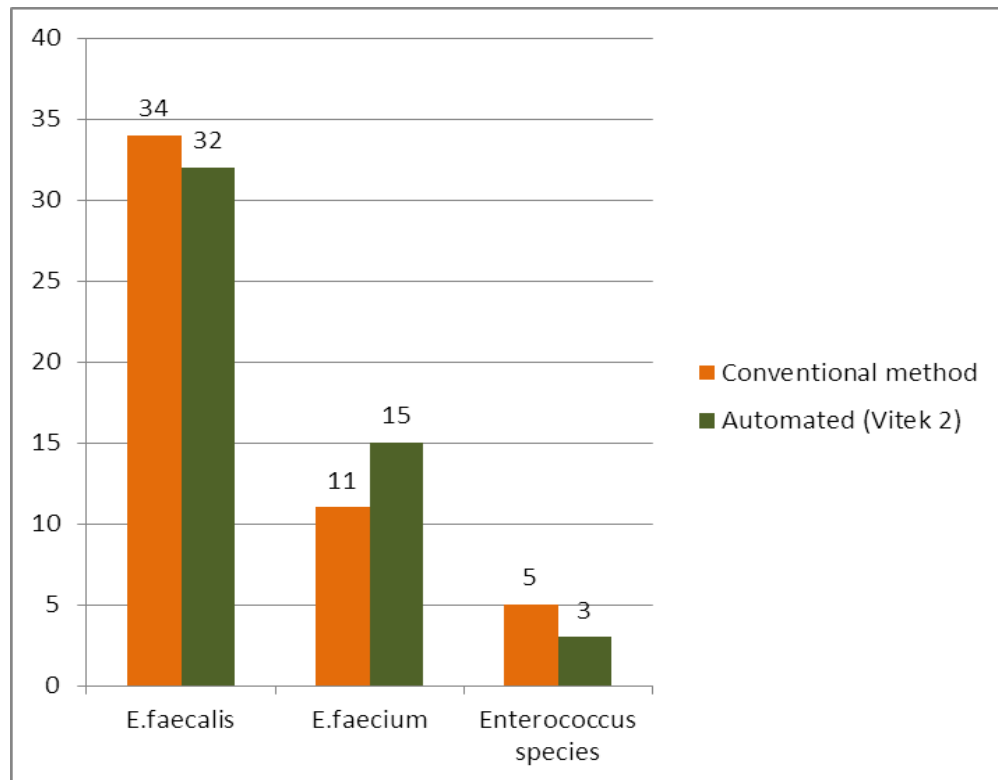


**Figure 2 – Speciation of *Enterococcus* from Clinical Isolates**

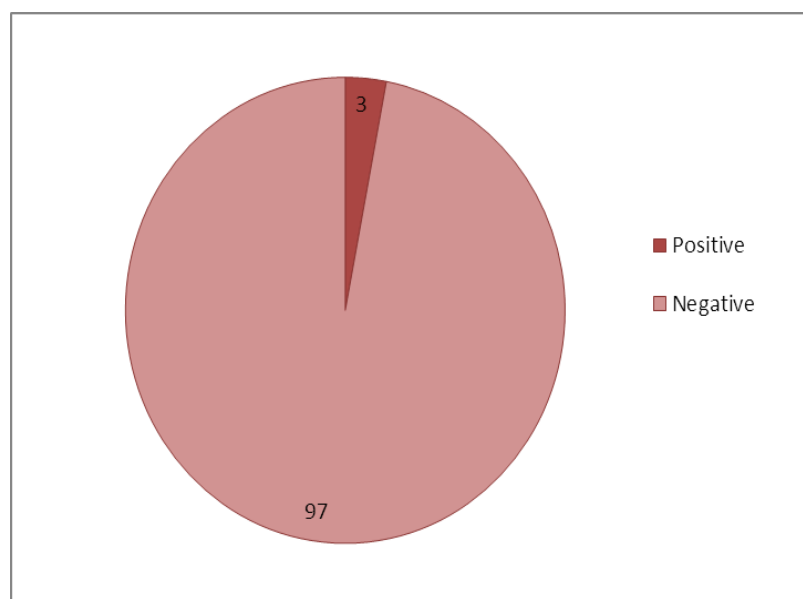


**Figure 3 – Comparison of *Enterococcus* Species Identification**

**N=50**

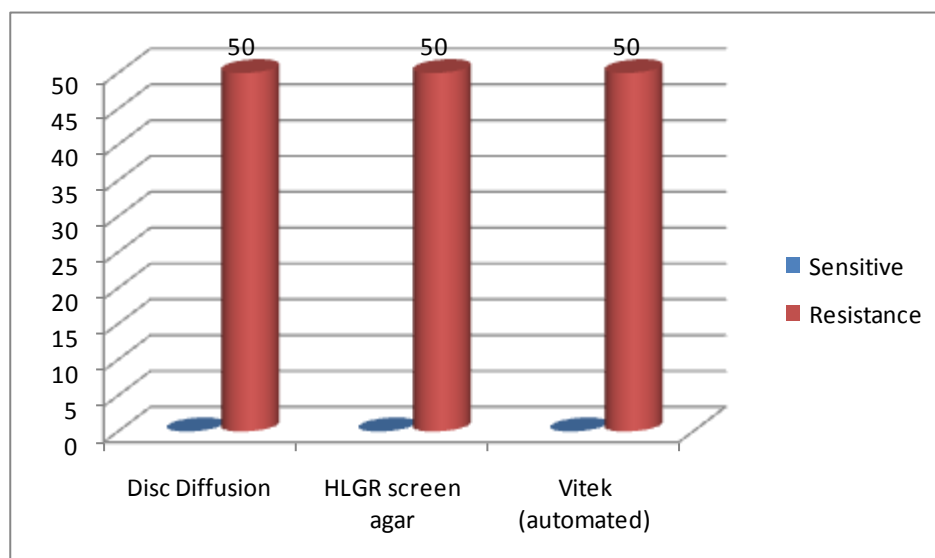


**Figure 4 – Screening of VRE carries from Stool**



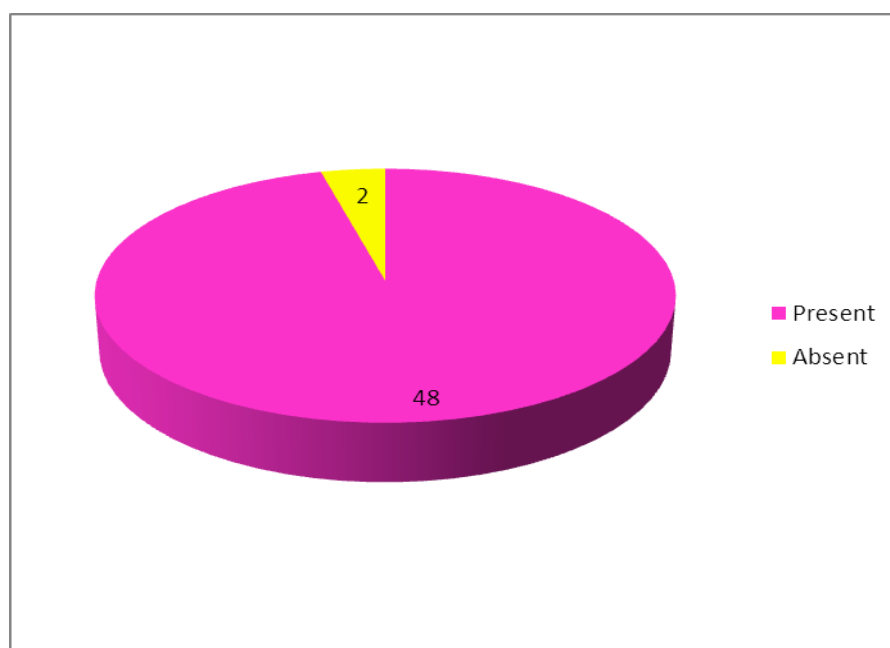
**Figure 5 – Comparison of Detection of resistant HLGR enterococcus**

**N=50**

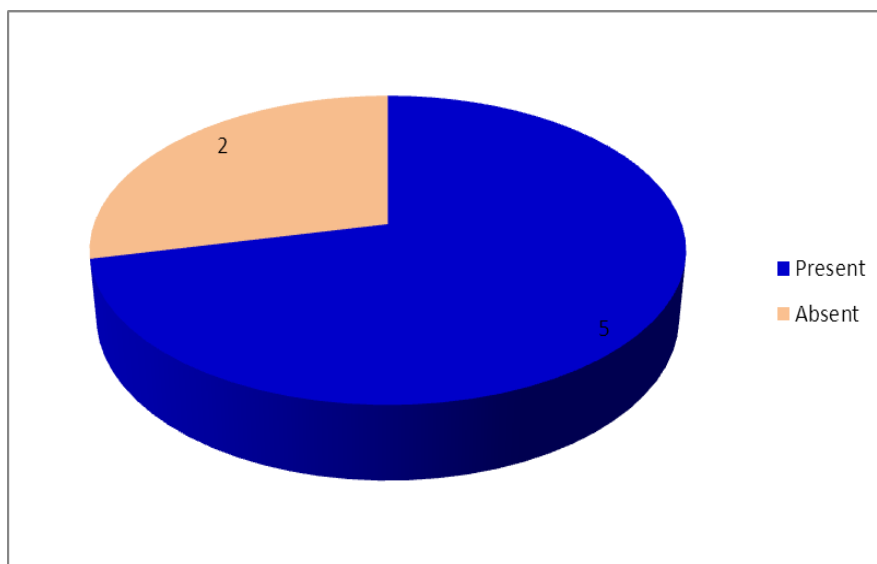


**Figure 6 – Detection of the presence of aac (6') le aph (2'') la gene**

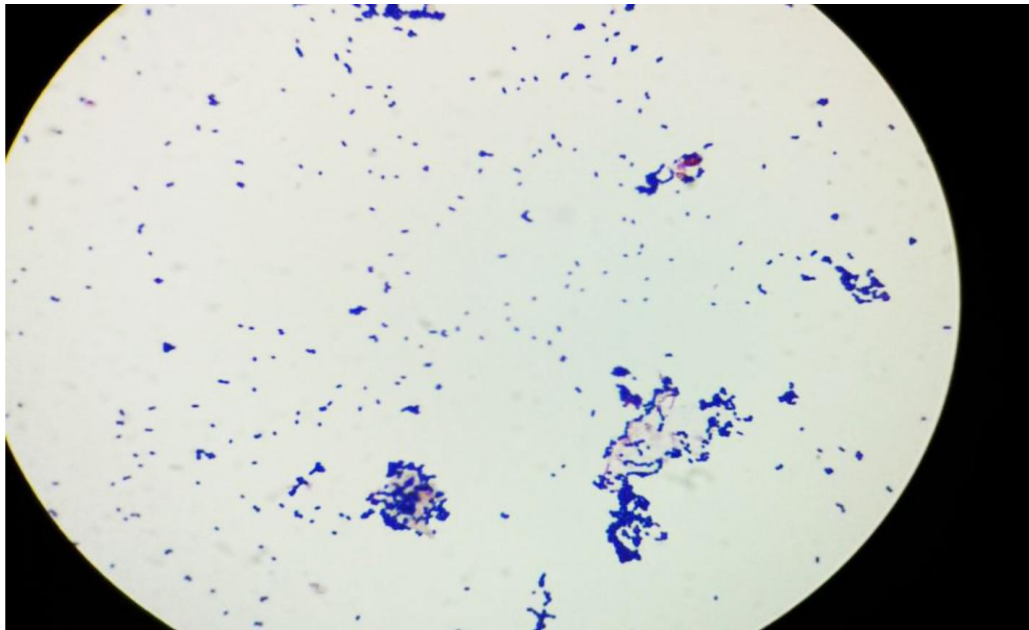
**N = 50**



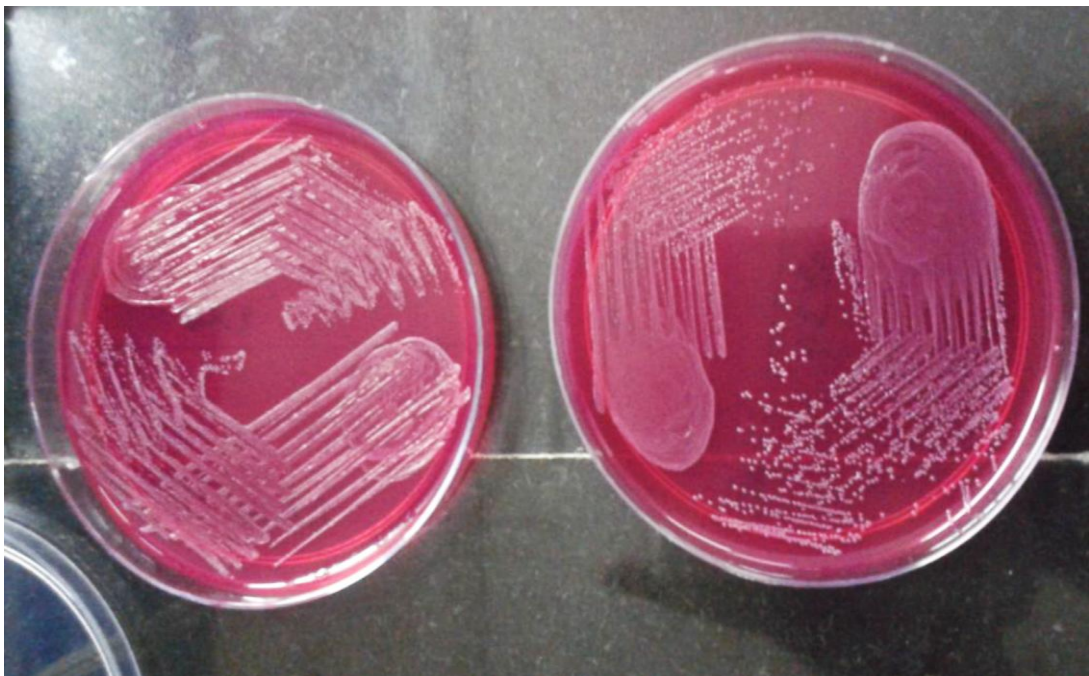
**Figure 7 – Detection of van A gene among the VRE isolates (N=7)**



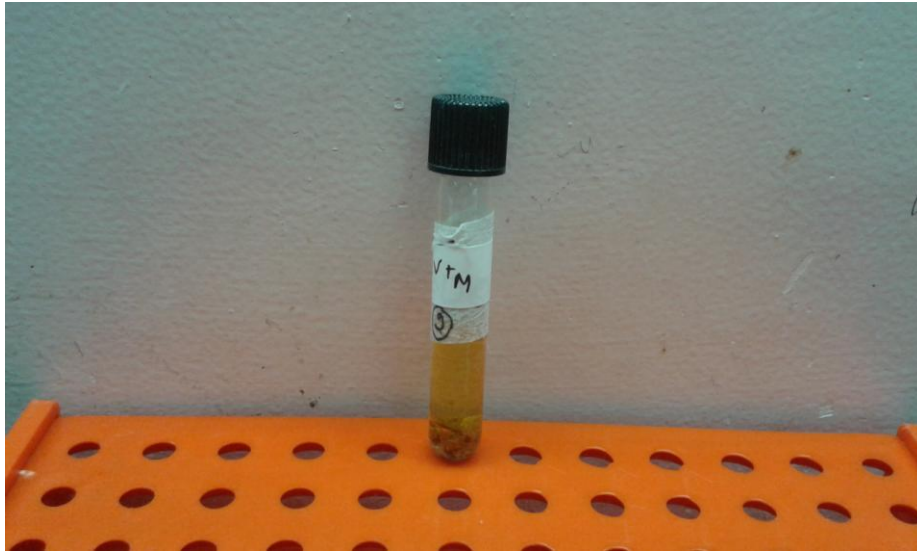
### ILLUSTRATION 1: GRAM STAIN



### ILLUSTRATION 2: Mac Conkey Agar plate

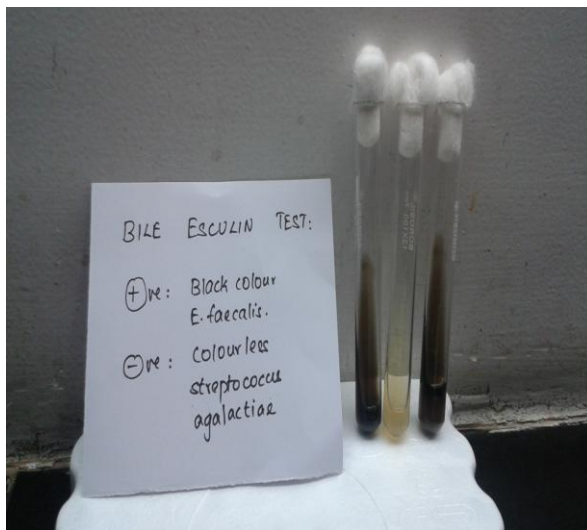


### ILLUSTRATION 3: ROBERTSON COOKED MEAT (RCM) MEDIA



### ILLUSTRATION 4: GENUS SPECIFIC TEST

#### A) BILE-ESCULIN TEST

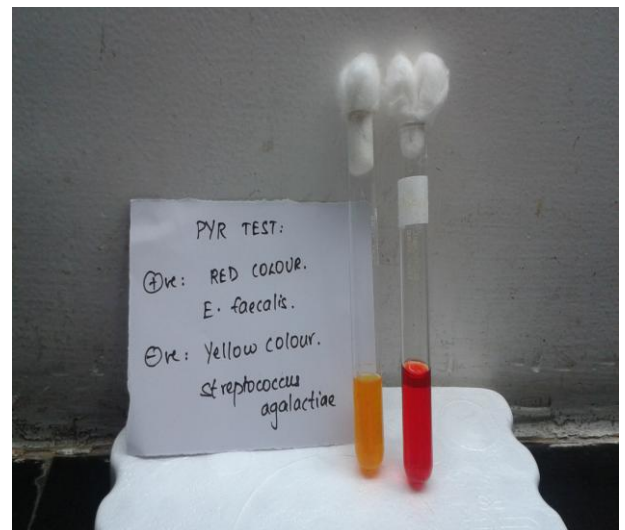


BILE ESCULIN TEST:

⊕ve: Black colour  
*E. faecalis*.

⊖ve: colourless  
*Streptococcus agalactiae*

#### B) PYR TEST



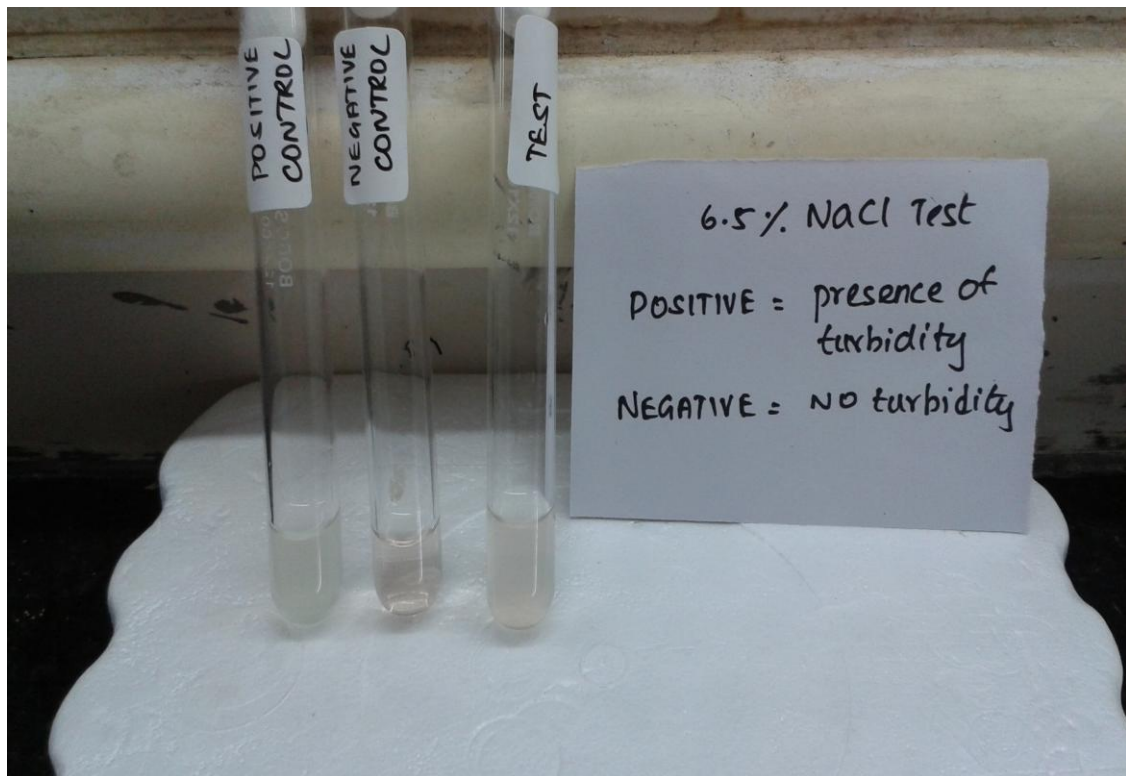
PYR TEST:

⊕ve: RED colour.  
*E. faecalis*.

⊖ve: Yellow colour.  
*Streptococcus agalactiae*

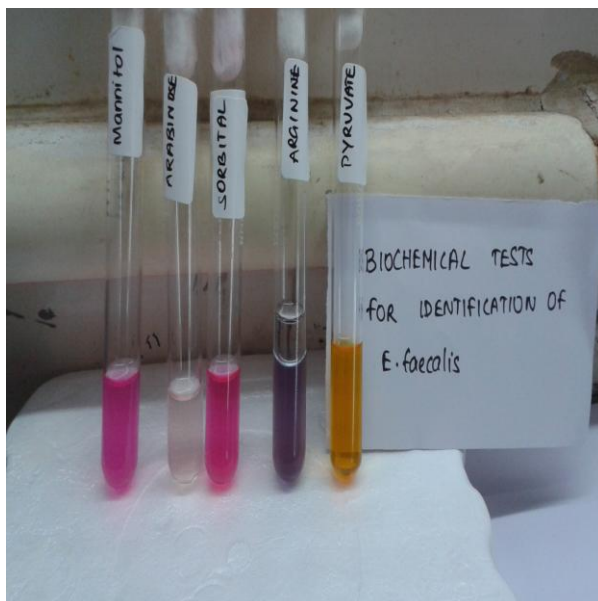


**C) 6.5% NaCl test:**



**ILLUSTRATION 5: SPECIES SPECIFIC TEST**

**A) *Enterococcus faecalis*:**



**B) *Enterococcus faecium*:**

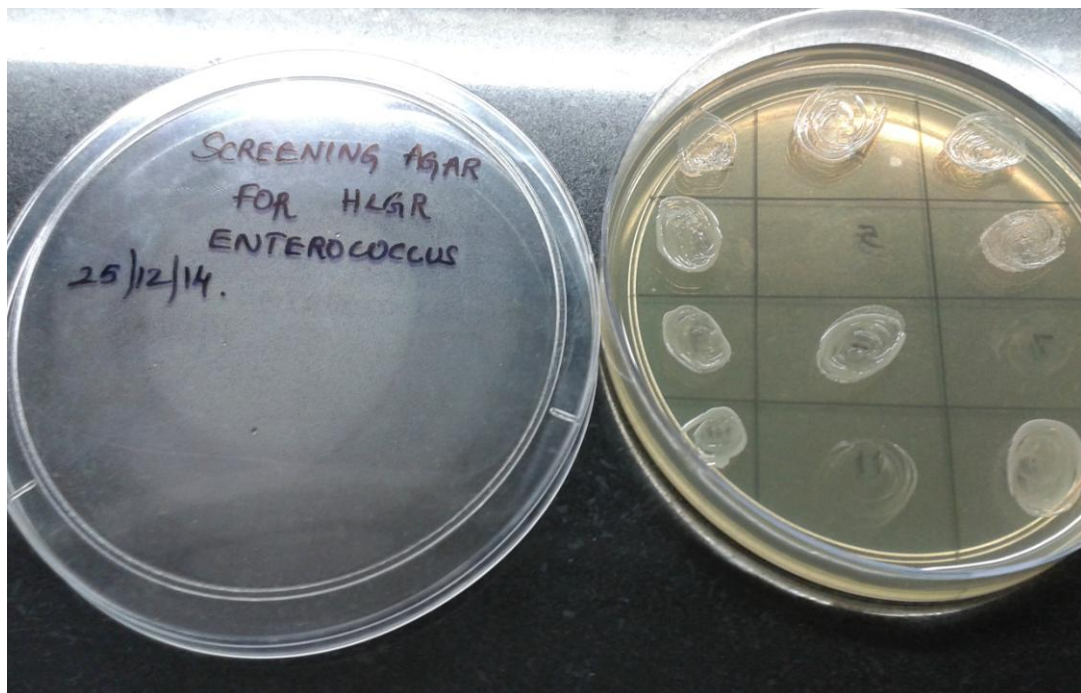




## ILLUSTRATION 6: KIRBY- BAUER DISC DIFFUSION TEST



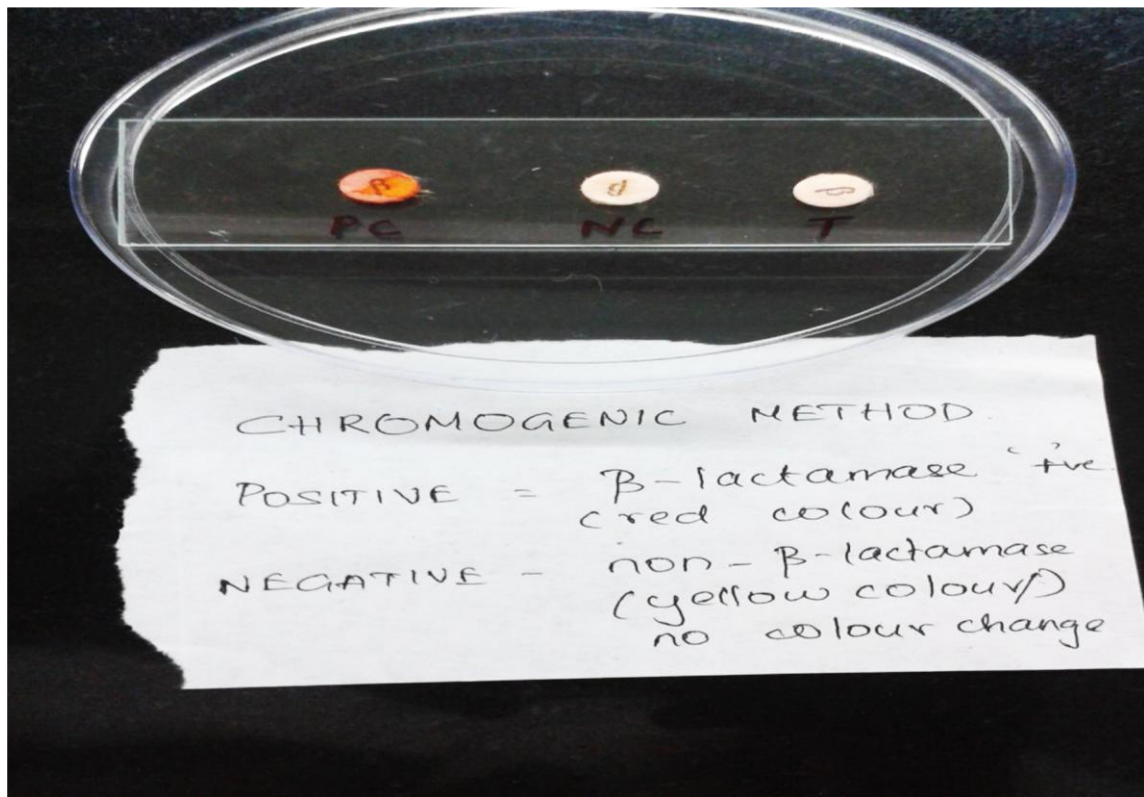
## ILLUSTRATION 7.A: HIGH LEVEL GENTAMICIN SCREEN AGAR



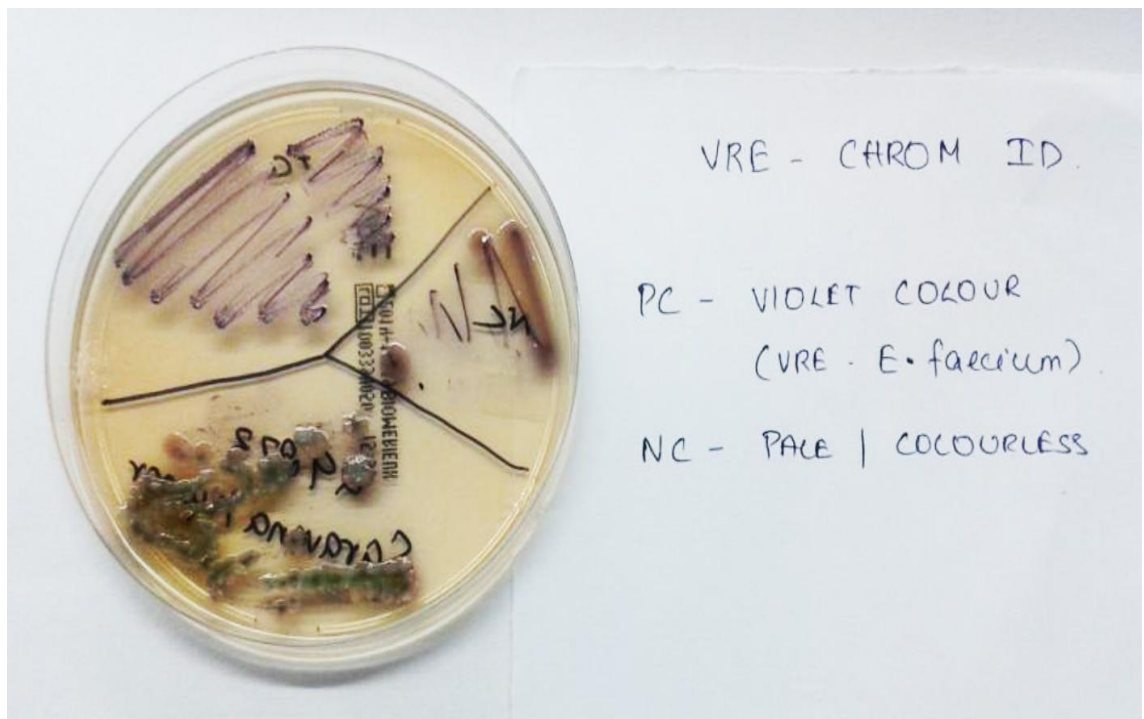
**ILLUSTRATION 7.B: HIGH LEVEL GENTAMICIN RESISTANT  
STRAINS**



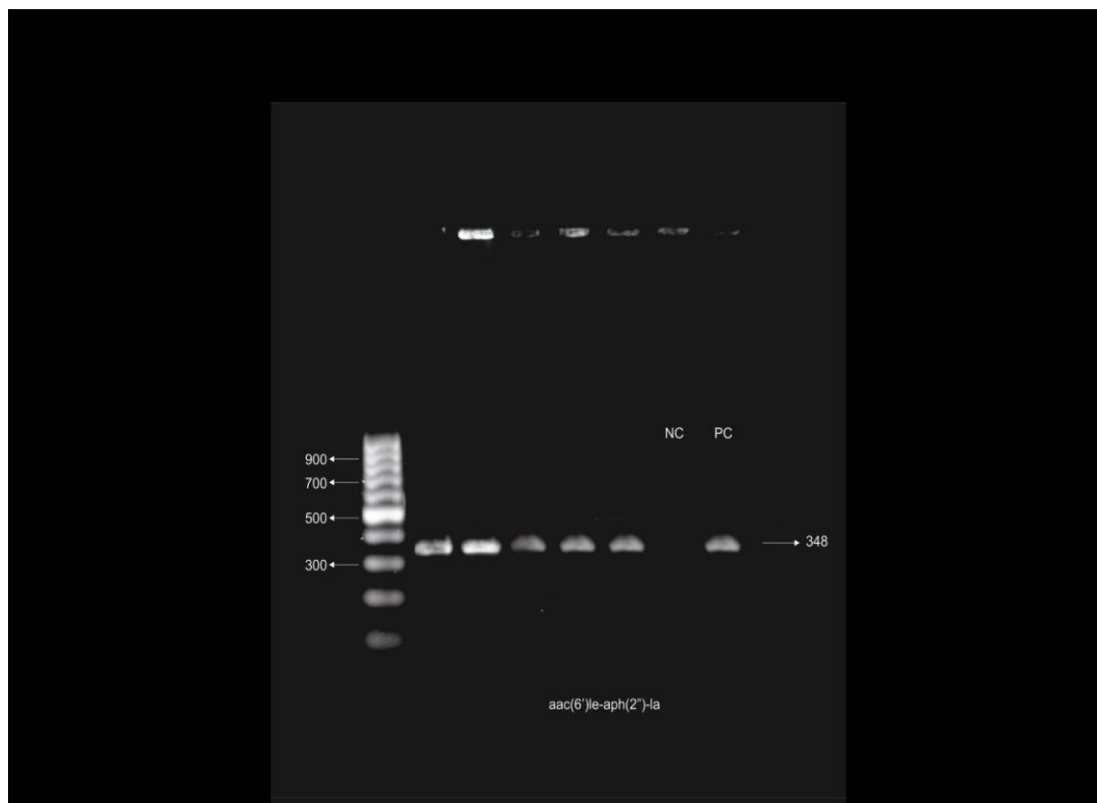
# ILLUSTRATION 8: BETA LACTAMASE DETECTION BY NITROCEFIDIN DISC



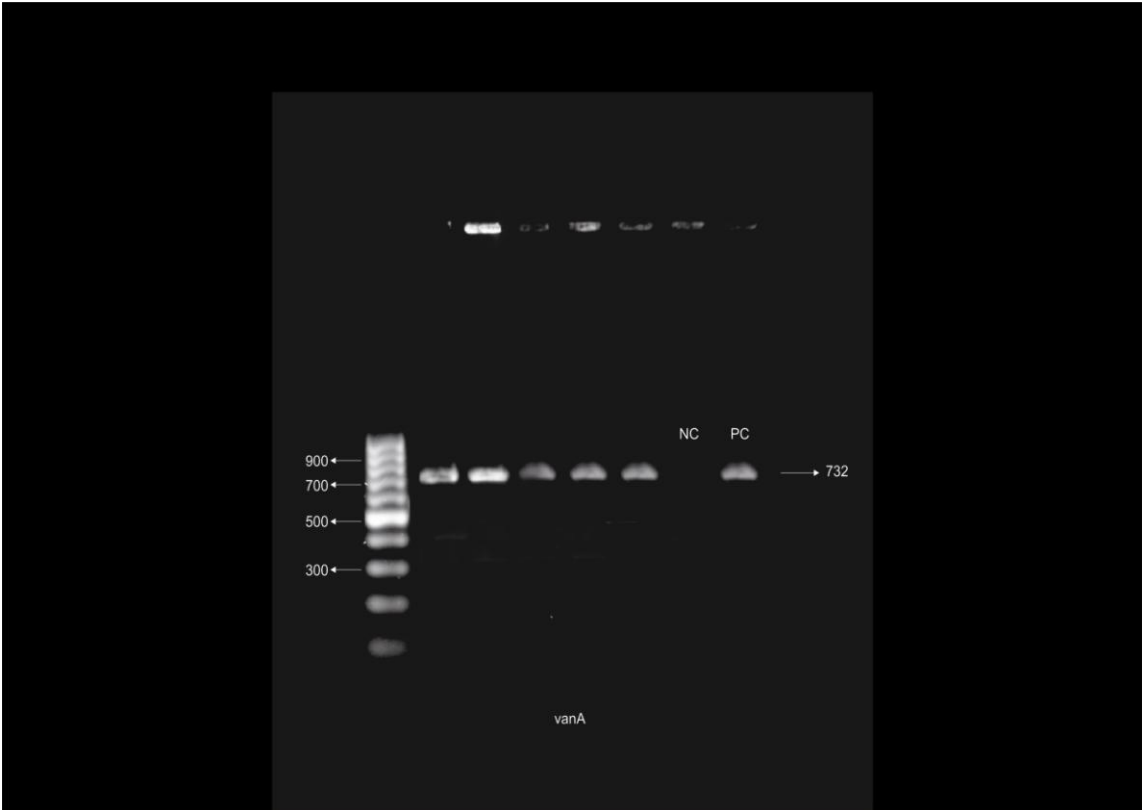
# ILLUSTRATION 9: VRE SCREEN AGAR



**ILLUSTRATION 10: PCR showing *aac(6')*-*aph(2'')* BAND OF SIZE 348 bp**



**ILLUSTRATION 11: PCR showing van A gene of band size 732bp**





## **ILLUSTRATION 12: MATERIALS REQUIRED FOR MOLECULAR STUDY**

### **GEL ELECTROPHORESIS**



### **GEL DOC FOR BAND VISUALIZATION**



## **DISCUSSION**

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*Enterococcus* has been noted to cause a majority of urinary tract infections. This being the most common followed by bacteremia, sepsis and even case reports of meningitis has been reported. In our study out of 250 samples studied the majority of the isolates were obtained from urinary tract infections (73.6%) followed by wound swabs (9.6%) and blood (5.6%). This correlates well with other studies in which *Enterococci* was obtained from urine isolates maximally.<sup>111,112</sup> According to the studies *Enterococci* are considered as the commonest urinary pathogen justifying the increase in the rate of isolation from urinary samples.<sup>8</sup>

Among the various species, in our study *E.faecalis* was the most common isolate accounting for 62.8% followed by *E.faecium* ( 31.6% ), *E.durans*(2%), *E.raffinosis*(2.8%) and 0.8% *E.casseliflavus*. Such findings were reported from other studies done in Bangalore in which *E.faecalis* was 59%, *E.faecium* was 38% .<sup>113</sup>

Identification of the enterococcal isolates by conventional methods and automated Vitek 2 Compact system was done which showed a sensitivity and specificity of 100% and 90% respectively. Studies done in a similar manner in comparison of manual and automated system (Phoenix) in identification of isolates showed 94.8% acceptability.<sup>114</sup> Another study done in Greece reported that there was no significant difference in the identification of the isolates by the two automated systems Vitek 2 and Phoenix.<sup>115</sup>



Enterococci show intrinsic resistance to cotrimoxazole, clindamycin, cephalosporins and normal aminoglycosides. Thus these antibiotics are not tested.<sup>77</sup>

The antibiotic susceptibility pattern of the isolates in our study showed the following:

The sensitivity to linezolid was maximum (98.8%) followed by vancomycin (97.2%), erythromycin (89.3%), teicoplanin (75.2%), ampicillin (69.2%), high level gentamicin (49.2%) and penicillin (25%). Among the urinary isolates maximum sensitivity was noted in Nitrofurantoin (92.3%) followed by Norfloxacin (83.15%). Studies done in North India showed a similar susceptibility pattern with increased resistance to penicillin and aminoglycosides. Also the sensitivity percentage to linezolid and vancomycin was higher compared to other antibiotics.<sup>116</sup> Another study showed the percentage of high level gentamicin resistance to be 68%.<sup>117</sup> Reports from South India showed the resistance percentage of high level aminoglycoside to be more than 50%.<sup>118</sup>

The screening of vancomycin resistant enterococci (VRE) carriers was done in 100 high risk patients in the intensive care units and immunosuppressed patients who were found to have a prolonged stay in the hospital. The incidence of VRE carriage was found to be 3%. In a similar study done elsewhere carrier rate was reported to be 6.6%.<sup>119</sup> Among the three patients identified as carriers, nephropathy followed by prolonged hospital stay and long term treatment with

antibiotics and diabetes was observed as the primary risk factors. The VRE carriers identified were isolated and appropriate precautions were advised in handling them. The resistant strains were identified to be *E.faecium* which correlated well with other studies that showed 100% of the carriers to colonise *E.faecium*.<sup>120</sup>

The chromogenic media used for the identification of carriers was reported to be more specific and convenient than the conventional biochemicals in the identification of resistant strains. VRE have been reported to cause serious nosocomial infections resulting in mortality and increase in the length of stay in the hospital. Rapid identification of these strains helps in early and appropriate management .<sup>119</sup>

Comparison of screening of resistant strains to high level gentamicin was done by disc diffusion, screen agar and Vitek 2 compact which showed a sensitivity and specificity of 100%. Similar comparative study showed 99% correlation.<sup>121</sup> High level gentamicin screen agar is comparatively cheaper and more efficient than disc diffusion and automated Vitek 2 system in identifying resistance. This method also has the advantage of testing many strains on a single agar plate. As there is an increase in the resistance rate exhibited by the isolates susceptibility to synergistic combinations with beta lactams is not effective in such isolates.<sup>8</sup> This could be used as a screening method in low cost settings where facilities like Vitek are not available.

The detection of beta lactamase production by the chromogenic nitrocefin disc method showed that none of the isolates had beta lactamase enzyme. Studies done in Delhi and in Maharashtra also showed similar results.<sup>122,123</sup> The resistance to beta lactams though common, the mechanism could be due to alteration in penicillin binding protein PBP 5 which is more common than beta lactamase production.<sup>88</sup>

The molecular study done to detect the presence of the resistant gene confirmed the presence of the bifunctional gene *aac (6')le-aph (2'')* of base pair 348 to be present in 48(96%) out of the 50 randomly selected resistant strains. Studies done in Chennai have showed the presence of this gene in 38.2% of the isolates<sup>124</sup> whereas there are other studies in which all the resistant isolates possessed this bifunctional gene.<sup>125</sup> The resistance to high level gentamicin indicates resistance to all the aminoglycosides except streptomycin.<sup>77</sup> The combination effect of aminoglycoside with beta lactam antibiotic is not helpful in the treatment of patients possessing such resistant strains.

Out of the 250 isolates the vancomycin resistant isolates obtained in our study was 2.8%. Studies have shown the prevalence of vancomycin resistance to be less in India ranging around 1.7-20%.<sup>118</sup>

Molecular studies revealed the presence of *van A* gene in 5(71.42 %) isolates.. Studies have also been reported showing *van A* to be the most common genotype isolated among the vancomycin resistant strains.<sup>98</sup>

Out of the seven isolates, five were *E.faecium* and two were *E.casseliflavus*. The remaining two isolates were *E.casseliflavus* which would have exhibited intrinsic resistance to vancomycin with van C gene.<sup>98</sup>

## **Summary & Conclusion**

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During the study period between April 2014-August 2015, out of the 250 isolates studied,

- 1) The incidence of enterococci from the clinical isolates in our study was 2.5%.
- 2) The incidence of the pure isolates was 86.4% in comparison to the mixed isolates which was found to be 13.6%.
- 3) Among the isolates of enterococci, the predominant species was *E.faecalis*(62.8%) followed by *E.faecium*(31.6%), *E.raffinosis*(2.8%), *E.durans*(2%) and *E.casseliflavus*( 0.8%).
- 4) The antibiotic sensitivity pattern of the urinary isolates showed 92.3% sensitive to nitrofurantoin and 83.15% sensitive to norfloxacin. Among the non-urinary isolates maximum sensitivity was observed in linezolid which was 98.8% followed by vancomycin (97.2%), erythromycin (89.3%), teicoplanin (75.2%), ampicillin(69.2), high level gentamicin sensitivity( HLG) (49.2%) and pencillin (25%).
- 5) Comparison of screening of resistant strains to high level gentamicin was done by disc diffusion, screen agar and Vitek 2 compact which showed that all the methods had equal sensitivity(100%) and specificity(100%)
- 6) The vancomycin resistance was found to be 2.8% in *E.faecium*.

- 7) All the isolates were found to be beta lactamase negative by chromogenic (nitrocefin) method proving the alteration in PBP5 to be the more common mechanism involved in penicillin resistance.
- 8) The molecular characterization of randomly selected 50 isolates ( out of 127) of HLGR (high level gentamicin resistant) strains revealed the presence of bifunctional gene(aac (6')le-aph (2'')) la to be 96%  
(Primers of base pair 348 - F (5'CAGAGCCTTGGGAAGATGAAG3')  
R (5'CCTCGTGTAATTCATGTTCTGGC3'))
- 9) The presence of van A gene was found to be 71.42% in our study.
- 10) Vancomycin resistant enterococcal( VRE) carrier rate was found to be 3% in this study.

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## **1) GRAM STAIN:**

### **Crystal violet:**

Crystal violet	1.0gm
5% sodium bicarbonate	1.0ml
Distilled water	99ml

### **Grams iodine:**

Iodine crystal	2.0gm
Sodium hydroxide	10.0ml
Distilled water	90.0ml

**Acetone-** 100%

**DILUTE CARBOL FUCHSIN-** 1 in 10 dilution.

## **2) BLOOD AGAR:**

Sterile defibrinated sheep blood    7ml

Nutrient Agar                                100 ml

About 7ml of melted Nutrient agar, as a base into sterile petri dishes and allow setting. Add sterile defibrinated blood(5-7%) to Nutrient Agar, the latter cooled to 45-50<sup>0</sup>C before blood is added. Mix and pour 20ml of blood agar in the petridish.

### **3) MAC CONKEY AGAR:**

Peptic digest of animal tissue	2.0gms
Lactose	1.0gms
Sodium taurocholate	0.5gm
Sodium chloride	0.5gm
Neutral red	1%
Agar	1.5gm
Distilled water	100 ml

Dissolve the ingredients except lactose in distilled water by heating. Adjust pH to 7.6. Sterilize by autoclaving at 121<sup>0</sup> for 15 minutes.

### **4) ROBERTSON COOKED MEAT MEDIA:**

Beef infusion broth.

Minced and dried meat.

One part of meat is mixed with two parts of water. Cool, refrigerate and skim off any remaining fat. Boil for 30 minutes. Filter through two layers of gauze and pH is adjusted to 7.5. Dried meat particles are distributed in 15\*150 mm tube to a height of 1.5 to 2.5cm. The filtrate is then added to get 3 to 4 parts. The tubes plugged and sterilized by autoclaving.



### **5) MEDIA WITH pH 9.6:**

Agar powder	1.5-1.8gms
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Nutrient broth	100ml
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Mix the agar in nutrient broth and heat to dissolve. When cool adjust the pH to 9.6. Sterilize by autoclaving. Pour 20ml in petridish. Dry the plate.

### **6) 6.5% Sodium chloride broth:**

Peptone	1gm
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Beef extract	0.5gm
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Sodium chloride	6.5gm
-----------------	-------

Glucose	0.1gm
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Distilled water	100 ml
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The above ingredients are mixed by boiling, Sterilized by autoclaving at 121<sup>0</sup> C for 15 minutes.

### **7) BILE AESCULIN AGAR:**

Beef extract	0.3gm
--------------	-------

Peptone	0.5gm
---------	-------

Bile salts	4gm
------------	-----

Aesculin	0.1gm
----------	-------

Ferric citrate	0.05gm
Agar	1.5gm
Distilled water	100ml
pH	7.0

The above ingredients are mixed by boiling and sterilized by autoclaving at 121<sup>0</sup> C for 15 minutes. Dispensed in tubes and allowed to cool in a slanted position.

#### **8) PYR media:**

Casein peptone	2.0gm
Beef heart infusion	0.3gm
Sodium carbonate	0.25gm
Dextrose	0.2gm
Sodium chloride	0.2gm
Disodium phosphate	0.04gm
L- pyroglutamic acid-beta naphthylamide	0.01gm
Distilled water	100ml
pH	7.8

The above ingredients are mixed and sterilized by autoclaving at 121<sup>0</sup>C at 15 minutes. These are dispersed in tubes and stored.

**9) ARGININE DECARBOXYLASE BROTH:**

Peptone	0.5gm
Beef extract	0.5gm
Bromocresol purple	0.001gm
Cresol red	0.0005gm
Glucose	0.05gm
Pyridoxal	0.0005gm
Distilled water	100 ml
pH	6.0

To the above 10gm of the L(levo) form of the amino acid arginine is added and dispensed in tubes. The above contents are autoclaved at 121<sup>0</sup>C for 15 minutes.

**10) POTASSIUM TELLURITE AGAR:**

Peptone	1gm
Sodium chloride	0.5gms
Beef extract	0.5gm

## **12) CARBOHYDRATE FERMENTATION MEDIA:**

Peptone water	100ml
Sugar	1ml
Andrade indicator	1ml

The above are distributed in tubes along with durhams tube and autoclaved at 115<sup>0</sup>C for 10 minutes.

The sugars used are mannitol, arabinose, raffinose, sorbitol, lactose and sucrose.

## **13) MULLER HINTON AGAR:**

Casein acid hydrolysate	1.75gm
Beef heart infusion	0.2gm
Starch	0.15gm
Agar	1.7gm
Distilled water	100ml
pH	7.3

The above ingredients are boiled to dissolve and sterilized by autoclaving at 121<sup>0</sup> C for 15 minutes .These are poured in petridish, dried and stored.

Agar	2gm
Potassium tellurite	0.04gm
Distilled water	100ml
pH	7.2

The basal ingredients are mixed by boiling, sterilized at 121<sup>0</sup>C for 15 minutes. To this potassium tellurite solution is added , mixed and poured in petridish.

#### **11) PYRUVATE MEDIUM:**

Tryptone	1gm
Yeast extract	0.5gm
Dipotassium phosphate	0.5gm
Sodium chloride	0.5gm
Agar	1gm
Sodium pyruvate	1gm
Bromothymol blue ethanolic solution: 4mg/ml	
pH	7.1-7.4

The solid ingredients are dissolved by heating, cooled and the indicator solution is added. The pH is adjusted and sterilized by autoclaving at 121<sup>0</sup> C. These are then dispensed in tubes and allowed to cool as slopes.

## **Annexures**

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# PSG Institute of Medical Sciences & Research

## Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)

POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA

Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

April 25, 2014

To  
Dr M Mohamadiya Rizwana  
Postgraduate  
Department of Microbiology  
PSG IMS & R  
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on April 11, 2014 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your study proposal entitled:

*"Isolation and characterization of Enterococcus from clinical samples in a tertiary care hospital"*

The following documents were received for review:

1. Duly filled application form
2. Proposal
3. Informed Consent forms
4. Confidentiality statement
5. Application for waiver of consent
6. Proforma
7. CV
8. Budget

After due consideration, the Committee has decided to approve the study.

The members who attended the meeting at which your study proposal was discussed are as follows:

Name	Qualification	Responsibility in IHEC	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
Dr P Sathyan	DO, DNB	Clinician, Chairperson	Male	No	Yes
Dr S Bhuvaneshwari	M.D	Clinical Pharmacologist Member - Secretary	Female	Yes	Yes
Dr Sudha Ramalingam	M.D	Epidemiologist Alt. Member - Secretary	Female	Yes	Yes
Dr Y S Sivan	Ph D	Member -Social Scientist	Male	Yes	Yes

The approval is valid for one year.



# PSG Institute of Medical Sciences & Research

## Institutional Human Ethics Committee

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POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA

Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

**We request you to intimate the date of initiation of the study to IHEC, PSG IMS&R and also, after completion of the project, please submit completion report to IHEC.**

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Yours truly,

  
**Dr S Bhuvaneshwari**  
**Member - Secretary**  
**Institutional Human Ethics Committee**





### ஒப்புதல் படிவம்

மரு.மு.முகம்மதியா ரிஷ்வானா ஆகிய நான் பி.எஸ்.ஜி. மருத்துவக் கல்லூரியில் நுண்ணுயிரியல் துறையின் கீழ் “மூன்றாம் நிலைத் துறை சேர்ந்த மருத்துவ மனையில் என்டிரோகாக்கை கிருமியை தனிமைப்படுத்தி அதன் இயல்பை ஆராய்தல்” என்ற தலைப்பில் ஆய்வு மேற்கொள்ள உள்ளேன். அதன் ஒரு பிரிவாக அவசர சிகிச்சைப் பிரிவில் உள்ள நோயாளிகளுக்கு வேன்கோமைசின் எதிர்ப்பு என்டிரோகாக்கை உள்ளதா என்று கண்டறிய உள்ளேன்.

என் ஆய்வு வழிகாட்டி : மரு. பார்வதி

#### ஆய்வு மேற்கொள்வதற்கான அடிப்படை

வேன்கோமைசின் எதிர்ப்பு என்டிரோகாக்கை உள்ளதா என்று கண்டறிந்து அது மற்ற நோயாளிகளுக்கு பரவாமல் தடுப்பதற்கு வழிகாட்டும்.

ஆய்வு மேற்கொள்ளும் நபர்களின் எண்ணிக்கை : இரண்டு

ஆய்வு மேற்கொள்ளும் இடம்

: பி.எஸ்.ஜி. மருத்துவமனை,  
பீளமேடு,  
கோவை.

இந்த ஆய்வில் பங்கேற்க ஒப்புக்கொள்ளுவதால் எந்த விதமான பலனும் உங்களுக்கு கிடைக்காது. எந்த நேரத்தில் வேண்டுமானாலும் ஆய்விலிருந்து விலகிக் கொள்ளும் உரிமை உங்களுக்கு உண்டு. ஆய்விலிருந்து விலகிக் கொள்ளுவதால் உங்களுக்கு அளிக்கப்படும் சிகிச்சையில் எந்தவித மாற்றமும் இருக்காது.

இந்த ஆராய்ச்சிக்காக உங்களிடம் பஞ்சுக்குச்சியை மலவாய் வழியாக செலுத்தி மலக்குடலில் உள்ள மலத்தை பரிசோதனை செய்யப்படும்.

மேலும், இந்த ஆய்வில் பங்கு கொள்வது உங்கள் சொந்த விருப்பம். இதில் எந்தவிதக் கட்டயாமும் இல்லை. நீங்கள் விருப்பப்பட்டால், இந்த ஆய்வின் முடிவுகள் உங்களுக்குத் தெரியப்படுத்தப்படும்.

ஆய்வாளரின் கையொப்பம்  
தேதி :

### ஆய்வுக்குட்படுவரின் ஒப்புதல்

நான் இந்த ஆராய்ச்சியின் நோக்கம் மற்றும் அதன் பயன்பாட்டினைப் பற்றி தெளிவாகவும், விளக்கமாகவும் தெரியப்படுத்தப்பட்டுள்ளேன். இந்த ஆராய்ச்சியில் பங்கு கொள்ளவும், இந்த ஆராய்ச்சியின் மருத்துவ ரீதியான குறிப்புகளை வரும் காலத்திலும் உபயோகப்படுத்தி கொள்ளவும் முழு மனதுடன் சம்மதிக்கிறேன்.

ஆய்வுக்குட்படுவரின் பெயர் :  
முகவரி

கையொப்பம் :  
தேதி :

### ஆய்வுக்குட்பவர் மிகவும் ஆபத்தான நிலையில் உள்ளது பொழுது ஆய்வுக்குட்பவரின் உறவினரின் ஒப்புதல்

நான் இந்த ஆராய்ச்சியின் நோக்கம் மற்றும் அதன் பயன்பாட்டினைப் பற்றி தெளிவாகவும், விளக்கமாகவும் தெரியப்படுத்தப்பட்டுள்ளேன். இந்த ஆராய்ச்சியில் எனது உறவினர் பங்கு கொள்ளவும், இந்த ஆராய்ச்சியின் மருத்துவ ரீதியான குறிப்புகளை வரும் காலத்திலும் உபயோகப்படுத்தி கொள்ளவும் முழு மனதுடன் சம்மதிக்கிறேன்.

ஆய்வுக்குட்படுவரின்  
உறவினரின் பெயர் :

முகவரி

கையொப்பம்  
தேதி :

ஆய்வாளரின் தொலைபேசி எண் :  
மனித நெறிமுறைக் குழு அலுவலகத்தின் தொலைபேசி எண் : 0422 2570170.

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*Enterococci* mainly being commensals in human faeces are now considered as an important cause of nosocomial infections. Of the infections, the commonly observed are urinary tract infections, abdominal infections followed by surgical site infections, bacteremia, endocarditis and meningitis sometimes. They are related to as the third organism in causing nosocomial infections in the west.<sup>1</sup>

*Enterococci* being the commonest micro flora of both humans and animals share their source of origin. They have been isolated from variety foods including cheese, fish, sausages, beef and pork. Studies have been reported that enterococci have been isolated from various clinical and environmental samples all over the world. Among the isolates obtained clinically it has been observed that *Enterococcus faecalis* has been the predominant species clinically.<sup>2,3</sup>

*Enterococcal* infections have been referred to as tough, tenacious and troublesome infections.<sup>4</sup> In the past years there has been an increase in the prevalence of *enterococcal* infections in hospitals and of particular concern is the emergence of antimicrobial resistant strains. Being second commonest organism behind the abdominal and pelvic infections, third in causing blood stream infections and also been reported in causing CNS and neonatal infections.

Reports have been confirmed with their association with clinical conditions including respiratory tract infections, osteomyelitis and cellulitis.<sup>5</sup> Enterococci initially regarded as the disease causing agent in the early nineties. But now various reports have been established considering them as the second commonest pathogen.<sup>6</sup>